A study of enzyme and sugar transport activities in T.b. bruceiplasma membranes

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A STUDY OF ENZYME AND SUGAR TRANSPORT ACTIVITIES

IN T.B. BRUCEI PLASMA MEMBRANES

Submitted by KEVIN CONROY

for the degree of Ph.D

of the University of Bath

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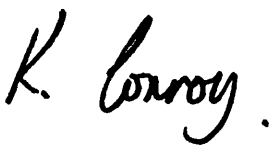

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SUMMARY

It was shown that T.b. brucei plasma membranes isolated after cell breakage with a tight fitting Dounce type homogeniser, were essentially free of cytosolic and mitochondrial contamination, but retained residual glycosomal contamination.

Dihydrolipoamide dehydrogenase (normally associated with pyruvate, 2-oxoglutarate and branched chain 2-oxoacid dehydrogenase complexes), was demonstrated to co-purify with the plasma membranes. The enzyme was released from its site of attachment by various detergents (but not by salt), and was readily reconstituted into lipid vesicles.

Three techniques; sodium hydroxide, calcium ion, and freeze/thaw treatments were applied to the plasma membrane vesicles. Only the freeze/thaw procedure produced vesicles with a fully functional D-glucose transporter.

Reconstitution experiments involving various techniques and detergents proved successful in reconstituting a fully functional erythrocyte D-glucose transporter and trypanosome dihydrolipoamide dehydrogenase. The trypanosome D-glucose transporter, however, could not be reconstituted in a functional form.

An infinite-trans sugar transport protocol was developed to study transport of D-glucose, 1-deoxy-D-glucose and 6-deoxy-D-glucose in the plasma membrane vesicles. Significantly higher Km values for D-glucose, 1-deoxy-D-glucose and 6-deoxy-D-glucose, but similar

Vmax values, were found in the plasma membrane vesicle preparation, compared to intact trypanosomes. Sugar transport inhibition studies suggested that there was spatial freedom around the carbon C2 position of the plasma membrane vesicle D-glucose transporter, and that the sugar binding requirements were less specific than those of the D-glucose transporter in the intact trypanosomes.

Covalent modification studies indicated that a tryptophan residue was required for an active D-glucose transporter. Dihydrolipoamide and NAD^+ (both substrates for dihydrolipoamide dehydrogenase) were found to alter the kinetics of D-glucose transport in the plasma membrane vesicle preparation. The NAD^+ effect could be negated by phenylarsine oxide. The possible involvement of dihydrolipoamide dehydrogenase in D-glucose transport is evaluated and discussed.

ABBRIEVIATIONS

ADP	Adenosine-5'-diphosphate
A-5-MP	Adenosine-5'-monophosphate
ATP	Adenosine-5'-triphosphate
CNS	Central nervous system
DDH_2O	Double distilled water
DHAP	Dihydroxyacetone phosphate
DHLIP DH	Dihydrolipoamide dehydrogenase
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNAase	Deoxyribonuclease
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
F-6-P	Fructose-6-phosphate
G-6-P	Glucose-6-phosphate
G-3-P	Glycerol-3-phosphate
KRB	Krebs Ringer phosphate buffer
MEGA 10	Decanoyl-N-methyl-glucamid
NAD^+	Nicotinamide adenine dinucleotide (oxidised form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
OL ATPase	Oligomycin sensitive $Na^+K^+Mg^{2+}$ stimulated ATPase

ABBREVIATIONS (continued)

OsO_4	Osmium tetroxide
OU ATPase	Ouabain sensitive Na^+K^+ ATPase
PBS	Phosphate buffered saline
PEP	Phosphoenol pyruvate
Pi	Inorganic phosphate
PPi	Pyrophosphate
SDS	Sodium dodecyl sulphate
SHAM	Salicylhydroxamic acid
TCA	Trichloroacetic acid
TEA	Triethylamine
Tes	N-tris[hydroxymethyl]methyl-2-aminoethane sulphonic acid
Temed	N, N, N', N-Tetramethylethylenediamine
Tris	Tris(hydroxymethyl) aminomethane
VSG	Variable surface glycoprotein
CMC	Critical Micelle Concentration

INTRODUCTION

1.0 GENERAL INTRODUCTION

Trypanosomes are flagellated protozoa about 10 to 30μm long responsible for many parasitic diseases of humans, domestic animals and wild animals, presenting severe problems both economically and medically to the Third world countries where they are prevalent. The World Health Organisation has rated Trypanosomiasis amongst the top six tropical diseases selected for study, with a view to developing more effective treatments (Trigg, 1979). Trypanosomes belong to the class Zoomastigophora, order Kinestoplastida, so named because they possess a mitochondrial associated kinetoplast. They are characterised by 5 main structural features (Fairlamb, 1982), fig. 1,

(1) a single flagellum,

(2) a complex skeletal array of subpellicular microtubules,

(3) microbody-like organelles called glycosomes, containing the first nine enzymes of glycolysis,

(4) a single but often highly branched mitochondrion containing,

(5) a dense network of DNA called the kinetoplast.

The kinetoplast distinguishes various stages of the organisms lifecycle, ie. trypanomastigote (kinetoplast posterior to the nucleus), and epimastigote (kinetoplast anterior to the nucleus), fig. 2.

Kinestoplastida display various lifecycles, from simple free living forms, the Bonidaes, to those that infect birds, mammals, fish, frogs, insects (Crithidia spp.),

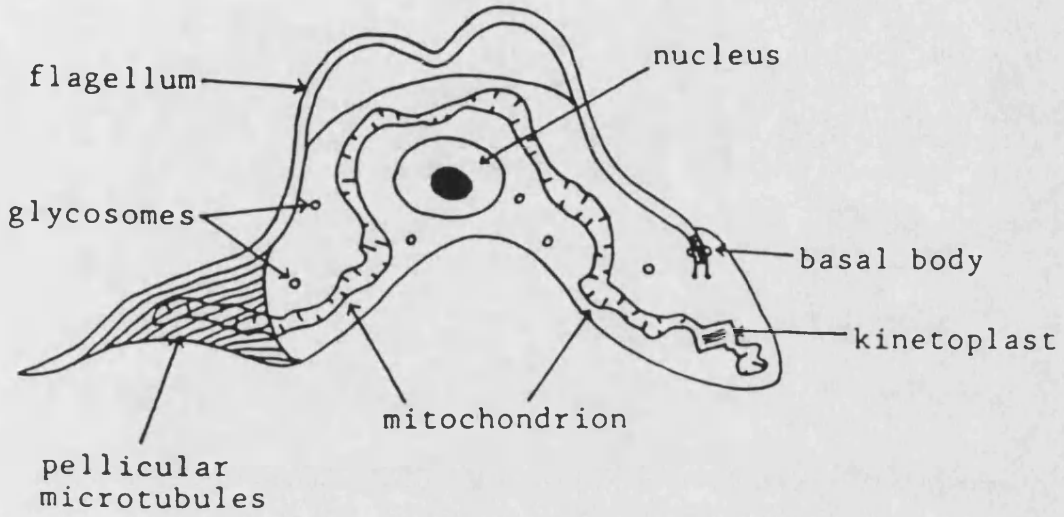
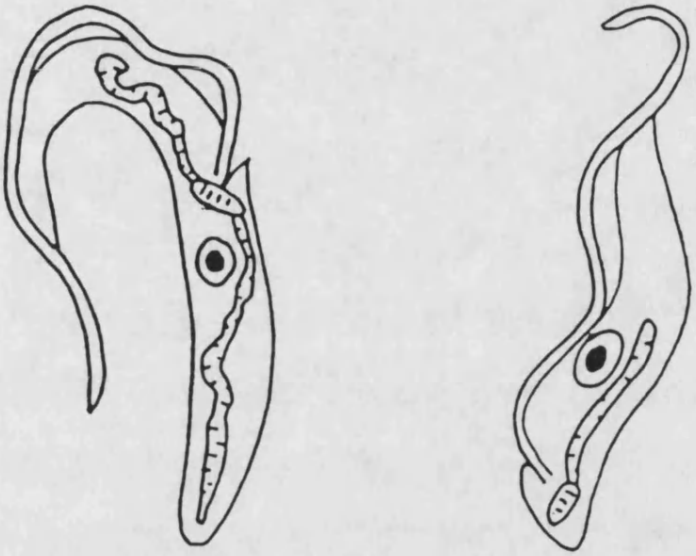


Fig. 1 The gross morphology of T.b. brucei (intermediate bloodstream form, Vickerman, 1965), showing the five main structural features.



Epimastigote
(Insect salivary gland)

Trypomastigote
(Mammalian bloodstream)

Fig. 2 Two examples of stages in the life cycle of Trypanozoon species showing the position of the kinetoplast (Newton, 1968).

and plants. Many of the infective species have complex life cycles involving a host vertebrate and an insect transmitting vector. The latter falls into two main groups, salivarian (salivary transmission) via blood sucking insects (flies, bugs and fleas) or stercorarian (faecal transmission). Uncommonly, mechanical transmission occurs via the mouth parts of biting insects in which the trypanosomes have a very short life. Exceptions include leaches which transmit the disease to fish and amphibians, the vampire bat (Desmodius rotundus) which has been implicated in the transmission of T. evansi and T. equiperdum , which has no intermediate host, transmission occuring during coitus (Jordan, 1986).

1.1 SOUTH AMERICAN TRYPANOSOMIASIS

The most important stercorarian trypanosome is T. cruzi, found in South America where it causes Chagas' disease. T. cruzi inhabits the Reduviid bug midgut (after ingestion in a blood meal) during the insect stage of its life cycle, fig. 3, and is excreted in the bug faeces. Infection of the host occurs by faeces entering the hosts skin via skin abrasions, through the mucous membranes, particularly the eye (Fairlamb, 1982), or by blood transfusions.

Trypanomastigotes penetrate the host cells of the infection site, where they transform into amastigotes which multiply within the host cells before being released into the blood stream as trypanomastigotes.

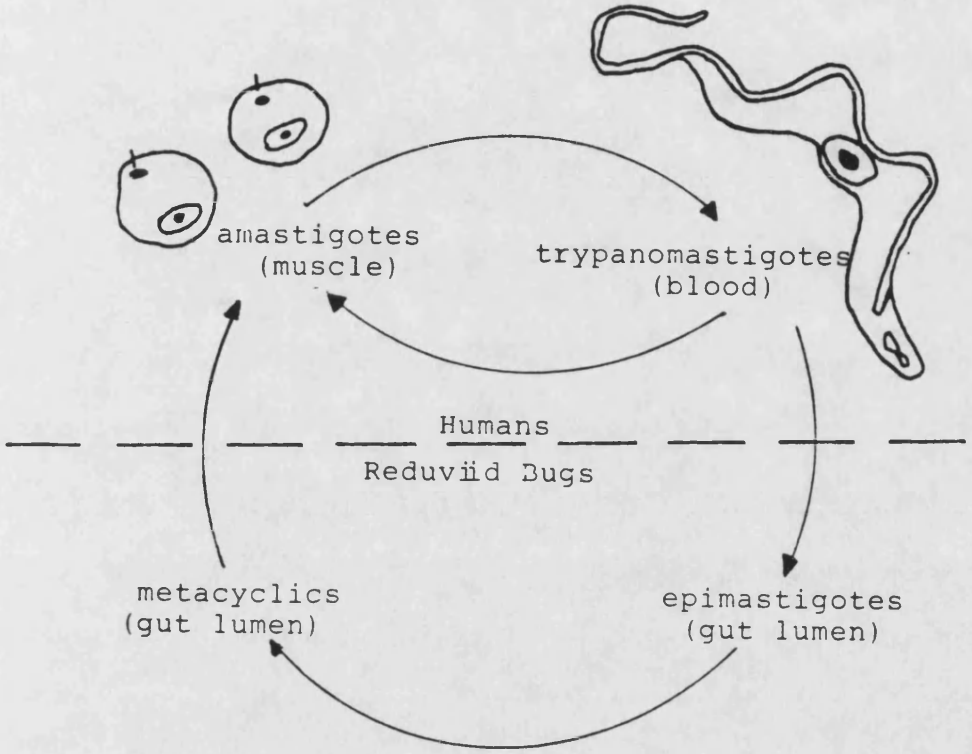


Fig. 3 Life cycle of T. cruzi (Fairlamb, 1982).

These non-dividing forms either penetrate new host cells particularly muscle, or are ingested during a blood meal by an uninfected reduviid bug during a blood meal. Unlike T.b. brucei there are no striking changes in morphology of the mitochondrion during the life cycle and their metabolism is essentially similar to the insect procyclic forms of T.b. brucei (Fairlamb, 1982).

In 1980 The World Health Organisation estimated that 35 million people were exposed to the infection, with 13 to 14 million carrying the disease. Untreated Chagas' disease is not inevitably fatal, but young children often die during the initial febrile stages (Fairlamb, 1982). Many adult victims survive the disease indefinitely but up to 20% of patients develop the final stage of the disease in which trypanosome antigens bind heart and nerve cells, stimulating an autoimmune response by the host, leading to the most common cause of death, congestive heart failure (Boreham, 1979). Current drug treatment is unsatisfactory with the two available drugs, a 5-nitrofuran derivative, and a 2-nitroimidazole derivative having such severe side effects that a full course of treatment is very rarely administered (Gutteridge, 1985). Toxicity and commercial considerations have halted the development of the most promising nitro-compound drugs.

1.2 AFRICAN TRYPANOSOMIASIS

In Africa the most important trypanosomes are

of the salivarian type transmitted by the Tsetse flies of the genus Glossina, to the normal vertebrate hosts, the large African wild mammals. These mammals generally show no pathogenic effects of the infection until stressed, and are thus able to harbour trypanosome infections for long periods of time, perhaps indefinitely, from which Tsetse flies acquire the trypanosomes. Four subgenera of salivarian trypanosomes are recognised, Table 1, however, the subgenus Pycomonus which contains only one species, T. suis, is very limited in distribution and importance. The three forms of the Trypanozoon subgenera, T.b. rhodesiense, T.b. gambiense and T.b. brucei, are thought to be identical both morphologically and biochemically (Hoare, 1964), however, some biochemical differences may exist. Epidemiological differences definitely exist, particularly with their infectivity to man, T.b. brucei being non-infective. It has been suggested by Vickerman (1965) that T.b. rhodesiense and T.b. gambiense are variants of T.b. brucei.

The human trypanosomiases (T.b. rhodesiense and T.b. gambiense), cause the fatal disease sleeping sickness which bears little in common with Chagas' disease. Up to 35 million Africans are at risk of catching the disease, with about 10,000 new cases reported each year (De Raadt, 1976), a figure that is almost certainly an underestimate because of the poor reporting conditions and medical services found in the affected areas. When medical surveillance and controls break down, as in Uganda (Gashumba, 1981), the disease reaches epidemic

Table 1 The tsetse-transmitted trypanosomes of African mammals (Jordan, 1986)

Subgenus	Species	Site of development in Glossina	Hosts	Distribution	Importance
Duttonella	T.vivax	Proboscis	Wild & domestic mammals (not pigs)	As for Glossina (Fig. 4)	Major disease of cattle & other ungulates
	T.uniforme	Proboscis	Wild & domestic mammals (not pigs)	East & Central Africa. Restricted.	Localised. Mild disease
Nannomonas	T.congolense	Proboscis & midgut	Wild & domestic mammals	As for Glossina	Major disease of cattle & other ungulates
	T.simiae	Proboscis & midgut	Wild & domestic pigs	As for Glossina	Acute disease of domestic pigs
Trypanozoon	T.brucei brucei	Salivary glands & midgut	Wild & domestic mammals	As for Glossina	Acute disease of dogs & horses. Chronic in cattle & pigs

(continued)

Table 1 (continued)

Subgenus	Species	Site of development in Glossina	Hosts	Distribution	Importance
Trypanozoon	T.brucei rhodiense	Salivary glands & midgut	Man & wild & domestic mammals	East & South-Central Africa	Acute form of sleeping sickness in man
	T.brucei gambiense	Salivary glands & midgut	Man. Wild & domestic mammals probably of some importance	West & North-Central Africa	Chronic form of sleeping sickness in man
Pycnomonas	T.suis	Salivary glands & midgut	Wild & domestic pigs	Tanzania, Burundi (? elsewhere)	Very localised Pathogenic to young domestic pigs

proportions.

Unlike Chagas' disease, African trypanosomiasis if left untreated always leads to death due to invasion of the central nervous system. The period of time until death is variable, from weeks to months, in the case of T.b. rhodesiense, following an acute course (Wakelin, 1984), to years in T.b. gambiense infections, following a chronic course.

In the acute form of the disease the first signs are a swollen chancre at the site of infection and/or a general oedema of the face. After several weeks an intermittent and irregular fever develops along with other general symptoms such as headaches, pains in the joints, loss of weight and severe toxaemia. Diagnosis of both forms may take several examinations because of the fluctuating nature of the disease. Chronic forms can be diagnosed by examination of cervical lymph node contents.

Invasion of the central nervous system is accompanied by epileptic fits, loss of apetite, and languor. Death is caused by many factors influenced by central nervous system deterioration, ie. respiratory failure, altered heart rhythm and beat, and by secondary infections caused by the immunosuppressive activity of the trypanosomes.

In animals, salivarian trypanosomes cause nagana in cattle (T.b. brucei, T. vivax and T. congolense) and surra in horses and camels (T. evansi), this leads to the death of over 3 million cattle every year making

it impossible to rear milk-producing cattle, sheep and goats in 10 million square kilometers of Africa (Fairlamb, 1982), fig. 4. Infection follows the same course as in humans. For instance, T. vivax and T. congolense can be acute with fulminating parasitaemia and death within two weeks of infection, or chronic showing no symptoms, lasting for months or years, with the acute stage brought on by stress. Wild animals generally have the chronic form.

The problems associated with trypanosomiasis have increased with attempts to eradicate the insect vector and wild animal parasitereservoirs failing, and increased drug resistance occurring.

1.3 LIFE CYCLE OF T.B. BRUCEI

T.b. brucei and other salivarian trypanosomes are injected into the host animal fly when the fly takes a blood meal. The lifecycle then follows the scheme shown in fig. 5 (Vickerman, 1985). Metacyclics injected into the host along with its salivary secretions are a heterogeneous population with a variety of glycoprotein coats. They multiply into trypanomastigotes at the bite site forming a chancre, before migration to the lymph nodes and then into the bloodstream. Once in the bloodstream they multiply rapidly invading the intracellular spaces of other tissues, including the central nervous system and cerebrospinal fluid.

The trypanosome's ability to evade the hosts

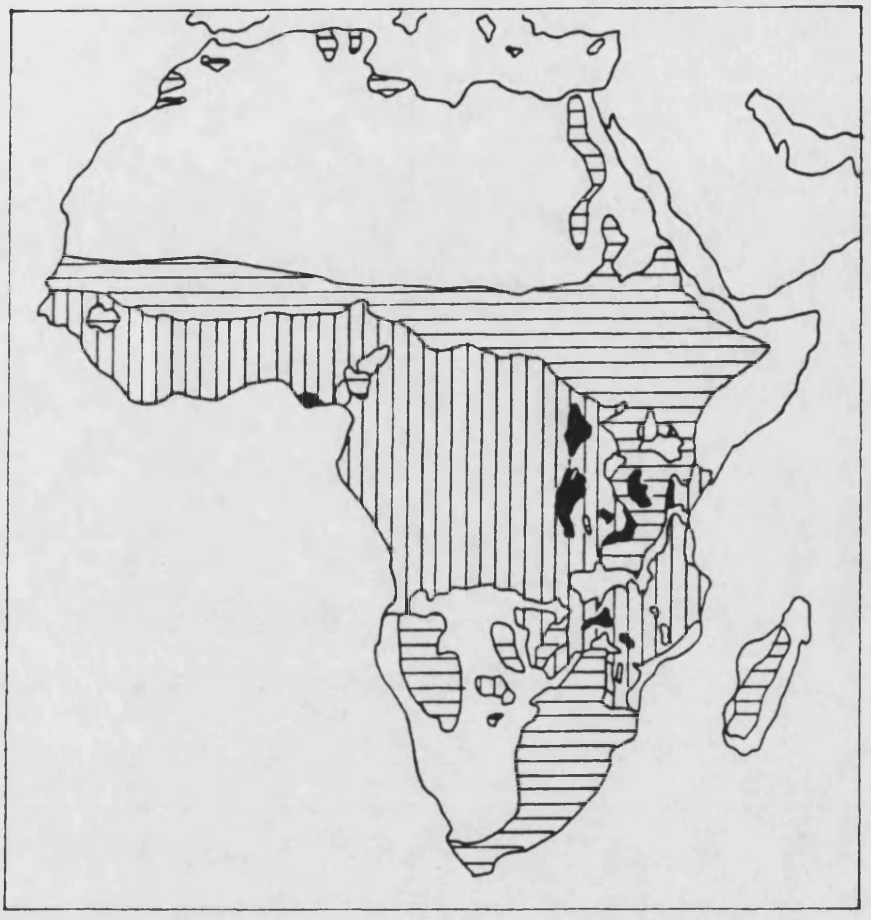


Fig. 4 Main cattle producing regions of Africa ▤ ,
Tetse fly areas of Africa ▥ ,
and areas where the two are found ■ ,
demonstrating virtual elimination of cattle from
Tsetse fly areas (Donelson & Turner, 1985).

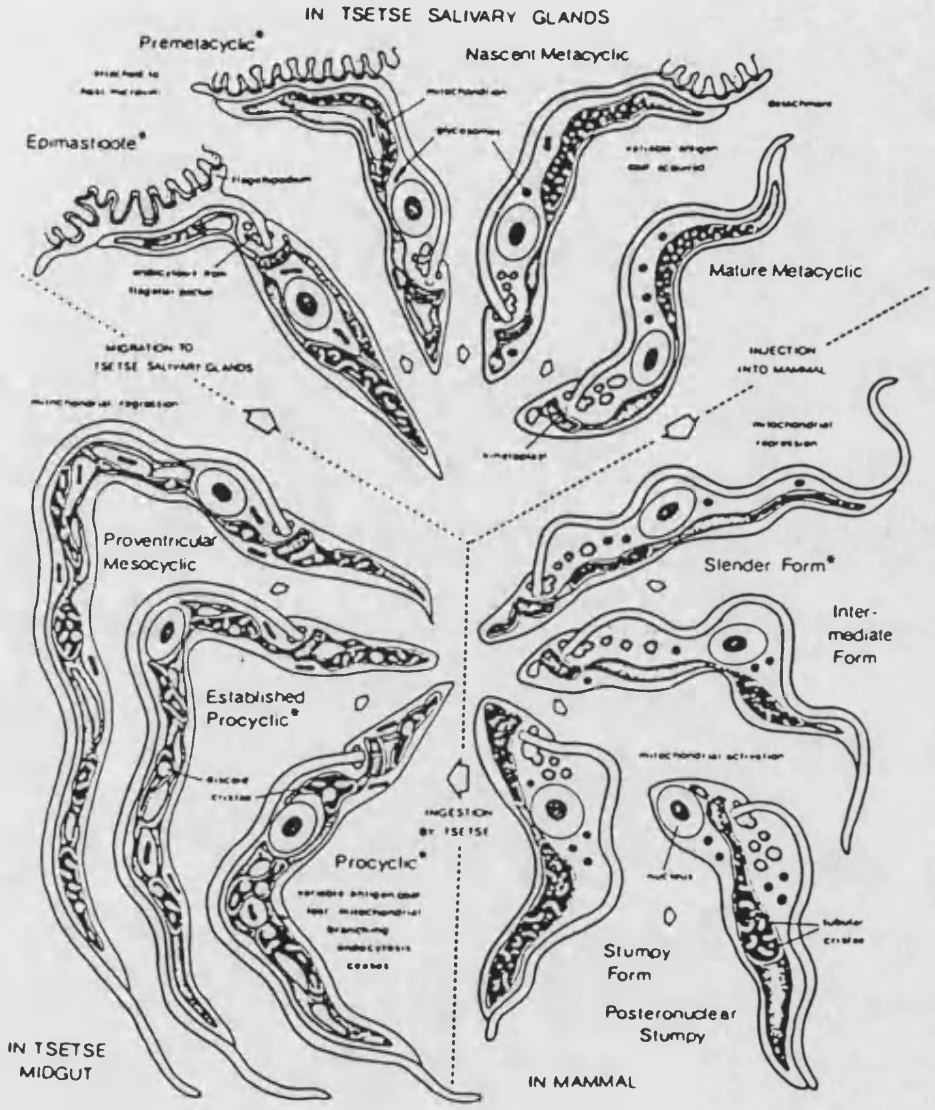


Fig. 5 Life cycle of the African trypanosome, T.b. brucei (Vickerman, 1985).

immune response arises from their ability to change their surface glycoproteins or VSGs.

Early in the course of an infection the immune system generates a complement-mediated response to the most common VSG on the surface of the invading organism, ensuring up to 99% of the organisms are killed. A few individual trypanosomes escape because they have turned on a different VSG gene and are covered by a new coat of VSG to which the available antibodies cannot bind (Donelson & Turner, 1985). These variants give rise to a new population expressing the new VSG of which up to 99% are ultimately killed by the host immune system. The remaining parasites multiply and trigger the host response. Once again the small number that have changed their protein coat survive and multiply. This gives rise to the undulating nature of the disease, fig. 6, ultimately leading to the host's death, if untreated.

In a chronic relapsing infection, bloodstream and tissue trypanosomes display various different morphological forms ranging from the long slender to the short stumpy forms. fig. 5. The short stumpy form is believed to be a preadaptation for survival in the insect vector. After ingestion by a Tsetse fly the short stumpy form transforms in the fly mid-gut into procyclic trypomastigotes, which multiply and migrate to the salivary glands, undergoing further transformations into epimastigotes and finally, infective metacyclic forms.

The life cycle is associated with many changes

in mitochondrial morphology and oxidative metabolism. In the bloodstream form the mitochondrion is a simple tube containing a few tubular cristae, no cytochromes, and cyanide insensitive respiration (Grant & Sargent, 1960; Flynn & Bowman, 1973; Fairlamb & Bowman, 1977). D-glucose is the sole energy source in the mammal and pyruvate the end product of aerobic metabolism, since the enzymes of the tricarboxylic acid cycle are absent. Transition to the short stumpy form involves the synthesis of TCA cycle enzymes including the pyruvate and α-ketoglutarate decarboxylases. Low levels of citrate synthetase, and succinate dehydrogenase, however, make the TCA cycle inoperative *in vivo* (Flynn & Bowman, 1973).

In axenic culture at 25°C bloodstream forms transform into an organism indistinguishable from the procyclic trypanomastigote. The mitochondrion becomes a complex branched network containing plate-like cristae (Vickerman, 1965; Bohringer & Hecker, 1974, 1975), synthesising cytochromes which become sensitive to cyanide under pure culture conditions. Proline, which is found in the insect hemolymph where D-glucose is in short supply, can replace D-glucose as the main energy source.

1.4 METABOLISM IN LONG SLENDER FORMS (fig. 7)

The bloodstream form is entirely dependent on D-glucose for energy supply. It has no energy reserves (Opperdoes *et al.*, 1976), and cannot respire on fatty

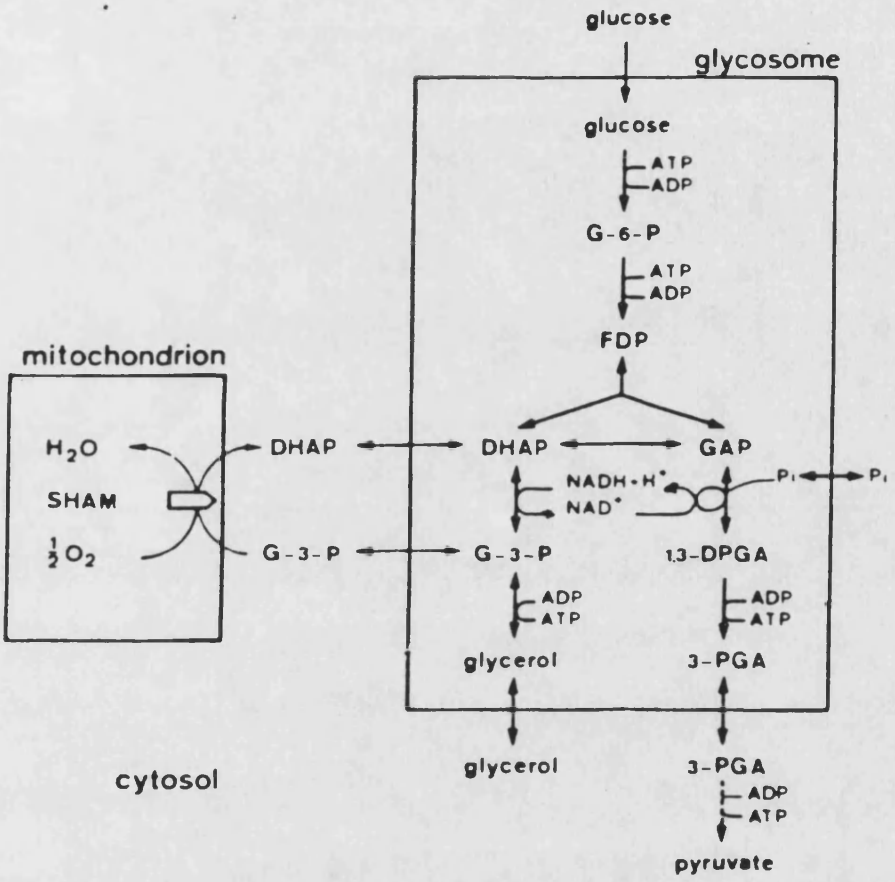


Fig. 7 Glycolytic pathway of T.b. brucei, showing the glycolytic activity and associated mitochondrial activity.

acids and amino acids (Bowman & Flynn, 1976). Aerobic respiratory activity is extremely high, about 60 to 90 nmol O_2/min/mg protein at 37°C, compared to 1 to 10 nmol O_2/min/mg protein, in mammalian cells.

Reoxidation of NADH produced during glycolysis in the glycosome is mediated through the activity of the mitochondrial sn-glycerol-3-phosphate oxidase (glycerophosphate oxidase), (Grant & Sargent, 1960; Opperdoes et al., 1977a, b; Fairlamb & Bowman, 1977). For each mole of triose phosphate converted to pyruvate a mole of NAD^+ is reduced to NADH which is reoxidised by a mole of DHAP being converted to glycerol-3-phosphate in the glycosome. Glycerol-3-phosphate is then reoxidised to DHAP by the glycerophosphate oxidase in the mitochondrion. This process forms the glycerophosphate oxidase shuttle, continually replenishing the NAD^+, and allowing all the D-glucose to be metabolised via glycolysis to pyruvate. Net ATP production in the glycosome is zero but the overall net production is 2 ATP per D-glucose molecule metabolised.

Under anaerobic conditions, or when SHAM is administered to inhibit the terminal oxidase, equimolar amounts of glycerol and pyruvate are produced (Ryley, 1956; Opperdoes et al., 1976), with D-glucose utilisation at the same rate as that found aerobically (Opperdoes et al., 1976). Unlike all the other glycolytic intermediates, L-glycerol-3-phosphate rises between 4-fold (Visser & Opperdoes, 1980), and 30-fold (Hammond & Bowman, 1980), giving high local concentrations in the glycosome.

Under these conditions the highly active glycerol kinase can act in reverse, catalysing the formation of glycerol and ATP from ADP and glycerol-3-phosphate. This hypothesis is supported by Gruenberg et al.(1980), who demonstrated that the Km for efflux of glycerol is lower than that for influx, suggesting an effluxing system for glycerol removal from the cell. The net ATP production is therefore one per D-glucose molecule.

1.5 TREATMENT OF AFRICAN TRYPANOSOMIASIS

Table 2 outlines the treatment of African trypanosomiasis.

The incidence of human trypanosomiasis is generally low and drug use correspondingly limited; in contrast some six million treatments are given annually to domestic animals, the vast majority for bovine trypanosomiasis (Jordan, 1986). In many African countries drug treatment is the only method of control, but its use is often under such inadequate supervision that drug resistance has evolved. No new drug for use in the field has been developed for many years.

Treatment of human African trypanosomiasis involves three main drugs, fig. 8; pentamidine in the prophylactic role, suramin and pentamidine in the early stages of the disease, and melarsoprol when the disease has spread to the CNS (Jordan, 1986).

Suramin is the most efficient in the prevention and early stages of the chronic form of T.b. gambiense

Table 2 Drugs for the treatment of African trypanosomiasis in man and domestic animals

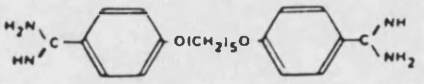
C = Curative; P = Prophylactic. (Jordan, 1986).

Drug	Trade name(s)	Action	Species mainly affected	Used especially in	Remarks
Tryparsamide		C	T.b.gambiense	Man	Removed from British & United States Pharmacopoeias
Melarsoprol	Mel B, Arsobal	C	T.b.gambiense T.b.rhodesiense	Man	Only drug available for treatment of patients with nervous system involvement
Nitrofurazone		C	T.b.gambiense T.b.rhodesiense	Man	Can be used in melarsoprol-resistant cases, but little used because toxic
Pentamidine	Pentamidine Lomidine	C,P	T.b.gambiense T.b.rhodesiense	Man	Only effective prior to nervous system involvement
Diminazene	Berenil	C	T.congolense T.vivax	Cattle,sheep, goats	Rapidly excreted, widely used

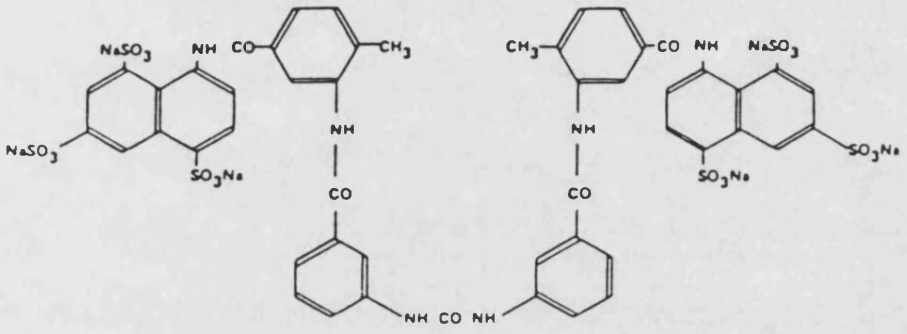
(continued)

Table 2 (continued)

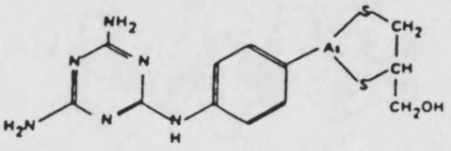
Drug	Trade name(s)	Action	Species mainly affected	Used especially in	Remarks
Homidium	Ethidium Novidium	C	T.vivax T.congolense	Cattle,sheep, goats	Widespread resistance
Pyrithidium	Prothidium	C,P	T.vivax T.congolense	Cattle,sheep goats	Resistance generally develops rapidly
Isometamidium	Samorin Trypamidium	C,P	T.vivax T.congolense	Cattle,sheep goats,horses	Widely used
Quinapyramine	Antrycide	C,P	T.congolense T.vivax T.b.brucei T.evansi	Cattle,sheep, goats,camels	Widespread resistance
Suramin	Antrypol, Naganol	C,P	T.brucei subspp, T.evansi	Man,camels, horses	In man only effective prior to nervous system involvement
Suramin-quinapyramine complex		C,P	T.simiae	Pigs	Restricted production. Not available commercially.



Pentamidine



Suramin



Melarsoprol

Fig. 8 Drugs currently used in the treatment of African trypanosomiasis.

infections, and is useful in the acute disease caused by T.b. rhodesiense. Suramin administration involves a thirty day intravenous treatment, compared to one week for pentamidine. The mode of action of pentamidine is thought to be by interruption of the synthesis of DNA, particularly in the kinetoplast. There are few side effects, but the drug is useless once the central nervous system is invaded, as it cannot cross the blood-brain barrier.

Suramin, a sulphonated napthylamine is a competitive inhibitor of glycerophosphate oxidase with respect to α-glycerophosphate. Its slow mode of action is a result of it being taken up by endocytosis as a protein bound complex (Fairlamb, 1982), and not by a transport process. Its effectiveness is limited by its inability to cross the blood-brain barrier.

Melarsoprol cures all stages of sleeping sickness because it is able to cross the blood-brain barrier. It is administered under strict hospital supervision because of the severe side effects, normally after the use of suramin which has a tonic effect. The trivalent arsenical has a high affinity for sulphydryl groups, particularly in kinases. Flynn and Bowman (1974),have demonstrated that pyruvate kinase is particularly sensitive, with inhibition resulting in a net zero ATP production by the cell, ultimately leading to its death. Where melarsoprol resistance occurs nitrofurazone, (which is even more toxic) is administered.

As recently as 1979 five main drugs were used

in the treatment of animals; homidium, quinapyramine, diminazene, pyrithidium and isometamidium. Since that time resistance has developed to all but isometamidium and diminazene. Isometamidium has prophylactic and curative properties, but diminazene is so rapidly eliminated from the animal that it has curative properties only. To overcome the drug resistance all of the above, particularly isometadium and diminazene, are used in combinations.

1.6 CARBOHYDRATE TRANSPORT IN T.B. BRUCEI AND THE RELATED STRAINS T.B. GAMBIENSE, T. LEWISI AND T. EQUIPERDUM

Much work has been published on the nature and function of glycolysis in the Trypanosomes, but the transport of carbohydrates across the plasma membrane of the cell has been poorly studied.

Early studies of carbohydrate transport were carried out on T.b. gambiense by Southworth and Read (1969 and 1970). D-glucose (Km 1.35mM, Vmax 47.9), D-mannose (Km 0.84mM, Vmax 48.3), D-glucosamine (Km 1.2mM, Vmax 28.5), N-acetyl-D-glucosamine, 2-deoxy-D-glucose (Km 0.16mM, Vmax 30.4), D-fructose (Km 1.58mM, Vmax 20.1, where Vmax is μmoles substrate/g dry weight/2min), were all transported by the trypanosomes, and, with the exception of N-acetyl-D-glucosamine followed saturation kinetics. D-glucose,D-mannose and glycerol transport were inhibited by each of the other 2 substrates, and each inhibited D-fructose. However, D-fructose did not inhibit the

transport of the other three. Both D-fructose and D-glucose inhibited D-glucosamine. Southworth and Read suggested two transport sites existed, one for D-glucose, D-mannose and glycerol, with a second for fructose, which was able to transport D-glucosamine.

Analogues of glycerol, D-glucose, D-fructose, various disaccharides and miscellaneous compounds were tested as inhibitors of glucose and fructose transport, but apart from those already mentioned, no inhibition was found (see Table 3). Glycerol analogues were, 1,3-propanediol, 1,2-propanediol, 1-propanol, lactic acid, propionic acid and glycerol. D-glucose analogues were, D-allose, D-galactose, L-fucose, D-sorbitol, D-mannitol, D-gluconic acid, D-gluconic acid lactone, glucuronic acid, glucuronic acid lactone, α-methyl-D-glucoside, phenyl-D-glucopyranoside and 3-O-methyl-D-glucose. Fructose analogues were, D-tagatose, L-sorbose; Disaccharides, cellulose, lactose, maltose and sucrose, and the miscellaneous compounds, inositol, ethylene glycol and D-arabinose.

The inhibition characteristics demonstrated a highly specific transporter, since analogues with only one altered hydroxyl group, ie. D-allose (C3), D-galactose (C4), and 3-O-methyl-D-glucose (C3) did not inhibit transport. C2 analogues including N-acetyl-D-glucosamine with a bulky side-group did inhibit transport, suggesting this position was less important for binding.

Sanchez and Read (1969) demonstrated transport of radiolabelled D-fructose, D-galactose, 3-O-methyl-D-glucose, D-mannose, D-glucosamine and D-glucose

Table 3 Carbohydrates tested as inhibitors of D-glucose and D-fructose transport in T.b. gambiense (Southworth & Read, 1970).

Inhibitor	Glucose	Fructose
A. Glycerol analogues		
1,3-propanediol	-	-
1,2-propanediol	-	-
1-propanol	-	-
Lactic acid	-	-
Propionic acid	-	-
Glycerol	+	+
B. Glucose analogues		
D-allose	-	-
D-galactose	-	-
L-fucose	-	-
D-sorbitol	-	-
D-mannitol	-	-
D-gluconic acid	-	-
D-gluconic acid lactone	-	-
Glucuronic acid	-	-
Glucuronic acid lactone	-	-
α-methyl-D-glucoside	-	-
Phenyl-D-glucopyranoside	-	-
3-O-methyl-D-glucose	-	-
D-glucose	+	+
D-mannose	+	+
D-glucosamine	+	+
N-acetyl-D-glucosamine	+	+
2-deoxy-D-glucose	+	+
C. Fructose analogues		
D-tagatose	-	-
L-sorbose	-	-
D-fructose	-	+
D. Disaccharides		
Cellobiose	-	-
Lactose	-	-
Maltose	-	-

(continued)

Table 3 (continued)

Inhibitor	Glucose	Fructose
D. Disaccharides		
Sucrose	-	-
E. Miscellaneous		
Inositol	-	-
Ethylene glycol	-	-
D-arabinose	-	-

(Km 1.25mM, Vmax 37.04μmols/g dry weight/10min), in Trypanosoma Lewisi, see Table 4. D-glucose was inhibited by all the sugars except D-glucosamine. D-fructose inhibited all of the sugars tested, particularly D-glucosamine, D-glucose and D-mannose. D-galactose, 3-O-methyl-D-glucose and D-mannose had their greatest effects on D-glucosamine, and lesser effects on D-glucose and D-fructose. D-glucosamine inhibited D-fructose and D-mannose (53%), whilst D-glucose inhibited D-fructose, 3-O-methyl-D-glucose, D-mannose and D-glucosamine, but not D-galactose. When D-glucose and D-glucosamine were combined at a concentration equivalent to the concentrations when used singly, inhibition of D-mannose rose to 88%.

Two transport sites explained these observations. Firstly a "D-glucose site" through which D-glucose was the preferential substrate and through which other sugars could enter. Secondly a "D-glucosamine site" for which D-glucosamine had the greatest affinity and D-fructose, D-galactose, 3-O-methyl-D-glucose, and D-mannose seemed to have a preference over the "D-glucose site".

The inhibition of transport of D-glucose, D-fructose, D-mannose and glycerol by a wide range of carbohydrates was carried out on T. equiperdum by Ruff and Read (1974). The carbohydrates tested included those used on Tb.gambiense by Southworth and Read (1970, Table 4), excluding lactic acid, propionic acid, D-allose, glucuronic acid, glucuronic acid lactone, cellulose and inositol. These were replaced by L-rhamnose, D-mannosamine, D-galacitol,L-fructose, D-ribose, D-xylose,

Table 4. % inhibition of the various hexoses on one another at a fixed inhibitor:substrate ratio of 8:1 . Taken from Sanchez and Read (1969). (All sugars are the D form).

Substrate (1mM) (C^{14} labelled)	Inhibitor (8mM)					
	Fructose	Galactose	3-O-methyl glucose	Mannose	Glucosamine	Glucose
Fructose	-	44(±17)	57(±20)	89(±15)	65(±15)	92(±1)
Galactose	0	-	0	0	0	0
3-O-methyl glucose	0	0	-	0	0	25(±11)
Mannose	39(±5)	21(±6)	43(±22)	-	46(±17)	86(±3)
Glucosamine	90(±1)	68(±6)	79(±6)	88(±1)	-	94(±2)
Glucose	55(±4)	50(±15)	56(±12)	79(±5)	0	1

Table 4 Reciprocal effects of the various hexoses on one another at a fixed inhibitor:substrate ratio of 8:1 . Taken from Sanchez and Read (1969). (All sugars are the D form).

Substrate (1mM) (C^{14} labelled)	Inhibitor (8mM)					
	Fructose	Galactose	3-O-methyl glucose	Mannose	Glucosamine	Glucose
Fructose	-	44(±17)	57(±20)	89(±15)	65(±15)	92(±1)
Galactose	0	-	0	0	0	0
3-O-methyl glucose	0	0	-	0	0	25(±11)
Mannose	39(±5)	21(±6)	43(±22)	-	46(±17)	86(±3)
Glucosamine	90(±1)	68(±6)	79(±6)	88(±1)	-	94(±2)
Glucose	55(±4)	50(±15)	56(±12)	79(±5)	0	1

D-xylitol, glucoheptulose, mannoheptulose, trehalose, D-propanol and glyceraldehyde, see Table 5.

The inhibition studies of C^{14} radiolabelled D-glucose, D-fructose, D-mannose and glycerol transport, were measured by incubation of the trypanosomes in 0.5mM radiolabelled substrate and 20mM inhibitor. Total radiolabelled sugar was measured in the trypanosomes after 2 minutes incubation.

The transport of D-glucose, D-fructose and D-mannose was inhibited by D-glucose, D-mannose, D-glucosamine, D-mannosamine, 2-deoxy-D-glucose, D-fructose, glycerol, glyceraldehyde, N-acetyl-D-glucosamine, 3-O-methyl-D-glucose, D-tagatose, mannoheptulose and maltose. Glycerol transport, however, was inhibited by the first eight of these carbohydrates but not the last five, see Table 5.

Ruff and Read (1974) concluded that three sites existed for carbohydrate transport. A hexose site, through which D-glucose, D-mannose and D-fructose were transported and two glycerol transport sites, site I and site II. Glycerol transport was inhibited by up to 80% by D-glucose (D-glucose:glycerol ratio 400:1), but never 100% unless unlabelled glycerol was added to the assay. Glycerol site I was therefore the hexose inhibitable site and site II the glycerol and glyceraldehyde inhibitable site, since both these compounds gave greater than 80% inhibition of glycerol transport.

The hexose transport site was distinct from glycerol site I because 3-O-methyl-D-glucose and N-acetyl-D-glucosamine inhibited D-glucose transport but not glycerol

Table 5 Effectiveness of various compounds as inhibitors of substrate transport in T.equiperdum (% inhibition) (Ruff & Read, 1974).

Inhibitor (20mM)	Substrate (0.5mM)			
	Glucose	Fructose	Mannose	Glycerol
Glucose analogues				
D-glucose	92.8	99.0	87.4	79.3
D-mannose	96.4	99.3	94.1	72.3
D-galactose	0	0	0	0
D-fucose	0	0	0	0
L-rhamnose	0	0	0	0
D-glucosamine	60.8	86.6	58.8	31.4
D-mannosamine	72.6	66.2	41.4	34.0
N-acetyl-D-glucosamine	72.3	84.8	67.4	0
N-acetyl-D-mannosamine	0	0	0	0
D-mannitol	0	0	0	0
D-galacitol	0	0	0	0
2-deoxy-D-glucose	99.0	100.0	99.2	48.8
3-O-methyl-D-glucose	63.1	58.2	51.0	0
D-gluconic acid	0	0	0	0
D-gluconic acid lactone	0	0	0	0
α-methyl-D-glucoside	0	0	0	0
Phenyl-D-glucopyranoside	0	0	0	0
Fructose analogues				
D-tagatose	30.8	33.8	31.3	0

(continued)

Table 5 (continued)

Inhibitor (20mM)	Substrate (0.5mM)			
	Glucose	Fructose	Mannose	Glycerol
Fructose analogues				
L-sorbose	0	0	0	0
L-fructose	80.2	90.7	82.0	62.9
D-sorbitol	0	0	0	0
Pentoses				
D-ribose	0	0	0	0
D-arabinose	0	0	0	0
D-xylose	0	0	0	0
L-xylose	0	0	0	0
D-xylitol	0	0	0	0
Heptoses				
Glucoheptose	0	0	0	0
Mannoheptulose	28.4	73.7	23.6	0
Disaccharides				
Sucrose	0	0	0	0
Lactose	0	0	0	0
Maltose	41.5	47.1	32.0	0
Trehalose	0	0	0	0
Glycerol analogues				
Glycerol	87.4	92.7	84.9	96.5
1-propanol	0	0	0	0
2-propanol	0	0	0	0
1,2-propanediol	0	0	0	0
1,3-propanediol	0	0	0	0
Ethylene glycol	0	0	0	0
Glyceraldehyde	95.9	55.8	89.3	35.7

transport. Inhibition of glycerol transport by D-glucose was caused by non-productive binding of D-glucose to the glycerol transport site without itself being transported. This conclusion was reached because D-glucose (0.1mM) transport was totally inhibited by 5mM phloridzin and glycerol transport by 89%. Addition of 0.1mM cold D-glucose inhibited the residual glycerol transport by 12.4%, under conditions where D-glucose was not transported. The inhibition of the D-glucose transporter by glycerol in intact trypanosomes has been studied by Game (1988) and in a plasma membrane vesicle system in this thesis. An explanation of glycerol inhibition of D-glucose transport is given in the discussion, section 6.1.

The carbohydrates transported by T. equiperdum and the inhibition of their transport by the inhibitors tested suggested that the hexose transport site could tolerate significant alteration of substituents at carbon C2 of D-glucose without affecting binding to the transporter. Carbon 4 was at least partially specific since D-galactose was not effective as an inhibitor. Carbons 1 and 6 could not be altered without affecting binding to the transporter.

The preceding transport characteristics of T.b. gambiense, T. lewisi and T. equiperdum can be summarised as follows. T. lewisi has two hexose transport sites, one with a high affinity for D-glucose and one with a high affinity for D-fructose and D-mannose (Sanchez & Read, 1969). T.b. gambiense has one site for D-glucose and D-mannose transport, with a second for D-fructose transport (Southworth & Read, 1970). T. equiperdum has

three transport sites, a D-glucose/hexose transport site and two glycerol transport sites (Ruff & Read, 1974). All three organisms have an ability to bind and transport D-glucose molecules altered at carbon 2.

Gruenberg et al. (1978) studied sugar transport in T.b. brucei, particularly the transport of D-glucose and 2-deoxy-D-glucose. They determined that D-glucose transport involved a carrier mechanism based on the following observations.

(1) The transport was saturable.
(2) Phloridzin a potent inhibitor of D-glucose transport, inhibited 2-deoxy-D-glucose transport.
(3) There was specific trans acceleration of efflux.
(4) L-glucose was not transported into the cells.
(5) p-Hydroxymercuribenzoate demonstrated a concentration-dependent inhibition of 2-deoxy-D-glucose transport.

D-glucose transport was not concentrative since the internal D-glucose concentration was always lower than the extracellular concentration of D-glucose, nor energy-dependent since under conditions where no ATP was produced (in the presence of salicyl hydroxamic acid), 2-deoxy-D-glucose penetrated the cells at a rate close to that of D-glucose in the absence of inhibitor.

Furthermore, D-glucose transport was the rate-limiting step in glycolysis. Gruenberg determined the maximum velocity of D-glucose transport over a period of one minute to be 25nmol/mg dry cells/min which approximates to 12.5nmol/10^8cells/min. This value compares with 50-100nmol/min/10^8cells (120-180nmol/mg protein/min) calculated by Flynn and Bowman (1973). Trypanosomal

hexokinase activity was calculated to be 790nmol D-glucose/ min/mg protein, in a whole cell homogenate of T.b. brucei by Opperdoes and Borst (1977c). Using the D-glucose transport/utilisation figures of Gruenberg et al. (1978) or Flynn and Bowman (1973), hexokinase activity is greater than D-glucose transport. Gruenberg et al. (1978) also determined that in efflux experiments greater than 50% of D-glucose and 2-deoxy-D-glucose was phosphorylated in the incubation time.

Gruenberg et al. (1978) suggested therefore that D-glucose was phosphorylated as soon as it entered the cell with the net uptake driven by the difference between intracellular and extracellular D-glucose concentration, ie. down a D-glucose gradient. The transport was therefore facilitated diffusion.

The most recent study of sugar transport in T.b. brucei by Game et al. (1986) utilised the D-glucose analogue, 1-deoxy-D-glucose.

The previously quoted transport studies have utilised various carbohydrates that have in some way been open to limited metabolism. Further to this, Visser et al. (1981) have pointed out that the high glycolytic flux poses a problem in interpreting uptake experiments since most of the results represent the steady-state rather than the initial rate. These problems could only be overcome using an efficient assay technique, and a D-glucose analogue not phosphorylated by hexokinase, nor metabolised by the cell.

Game et al. (1986) demonstrated that 1-deoxy-D-glucose gave no detectable ADP production by hexokinase

in a coupled system (<0.1% of the rate with D-glucose) at concentrations up to 10mM. At concentrations of 10mM 1-deoxy-D-glucose and 0.25mM D-glucose, no inhibition of D-glucose activity with hexokinase could be detected. Oxidation of D-glucose by the trypanosomes was inhibited by 1-deoxy-D-glucose presumably at the level of the membrane transporter.

1-Deoxy-D-glucose influx followed Michaelis-Menten kinetics with a Km of 4.03mM and Vmax 0.052mMs^{-1}. D-glucose was a competitive inhibitor of 1-deoxy-D-glucose transport with a Ki of 0.33mM. In contrast the rate of 3-O-methyl-D-glucose uptake into the trypanosomes was less than 10% of the rate of 1-deoxy-D-glucose uptake.

2.0 RECONSTITUTION OF MEMBRANE PROTEINS

Reconstitution involves the incorporation of proteins or protein complexes into phospholipid or lipid mixtures containing phospholipids, to form bilayers with inserted proteins, as either vesicles or planar membranes. It may or may not involve the use of detergents.

Incorporation of membrane proteins involves two kinds of proteins. Firstly the incorporation of a peripheral protein that relies on a lipid mixture for stability, orientation etc. These proteins can often be dissociated using chelating agents, lowering or increasing ionic strength and pH, without disrupting the membrane structure, eg. cytochrome c. The proteins do not span the membrane and have non-vectoral activity. In the microsomal electron transfer chain, the amphipathic proteins cytochrome b_5 and NADH-cytochrome b_5 reductase, (Rogers & Strittmatter, 1973 , Dailey & Strittmatter, 1981), have a hydrophilic moiety free in solution and a hydrophobic one anchored in the membrane. In contrast to the transmembrane proteins, insertion occurs with no demand for either detergents or specific lipids, the amount of insertion relying primarily on available membrane area.

The second type of protein spans the membrane matrix, permitting the translocation of solutes and possibly generating chemical and potential gradients, ie. the mitochondrial proton translocating ATPase

(Kagawa & Racker, 1971). These proteins very often require detergents, specific lipids and various techniques for reconstitution.

Reconstitution is an important technique in the study and elucidation of function of transmembrane proteins since it provides the membrane matrix for protein orientation and activity, and the production of separated compartments to allow the monitoring of solute exchanges between the two faces of the bilayer.

The D-glucose transporter fits into the second category of membrane proteins; hence reconstitution of transmembrane proteins will be stressed.

2.1 TECHNIQUE FOR PROTEIN RECONSTITUTION

2.1.1 Detergent dialysis and dilution reconstitution procedures

Detergent dialysis using cholate was the original method of reconstitution, developed by Kagawa & Racker (1971), for the reconstitution of the Na^+K^+ ATPase of bovine heart mitochondria. The technique has since been applied to other proteins and detergents. The protein or mixture of proteins is extracted by high critical micelle concentration detergents such as sodium cholate and n-octyl-β-D-glucopyranoside. The detergent and protein extract is mixed with an excess of phospholipid forming a suspension of mixed micelles containing protein, lipid and detergent, lipid and detergent, and protein and detergent. Detergent is removed by dialysis over a

period of 12 to 48 hours, allowing proteoliposomes containing the transport protein to "self assemble" as shown in fig. 9.

Detergent dialysis has been applied successfully with sodium cholate and more recently n-octyl-β-D-glucopyranoside in the reconstitution of the D-glucose transporter of red blood cells by Shelton and Langdon (1983). A refinement of the technique replaces dialysis of the detergent-phospholipid-protein mixture by rapid dilution with appropriate buffer, resulting in a detergent concentration, which, if not negligible, is significantly below the critical micelle concentration.

Detergent dilution has been applied to reconstitution of the Ca^{2+} pump of sarcoplasmic reticulum by Racker et al. (1975) and the asymmetric insertion of the phage M13 coat protein of E.coli by Racker (1979) using sodium cholate. More recent applications of the dilution procedure replace cholate with n-octyl-B-D-glucopyranoside. Newman and Wilson (1980) solubilised and reconstituted the lactose transport system of E. coli and Chia-Chen Chen et al. (1986) solubilised and reconstituted the erythrocyte D-glucose transporter.

Fig. 10 demonstrates a possible system for protein incorporation by detergent dilution (Eytan, 1982).

2.1.2 Physical reconstitution procedures

Physical reconstitution methods involve primarily sonication and freeze-thaw techniques. Sonication was the original reconstitution method which involves sonicating

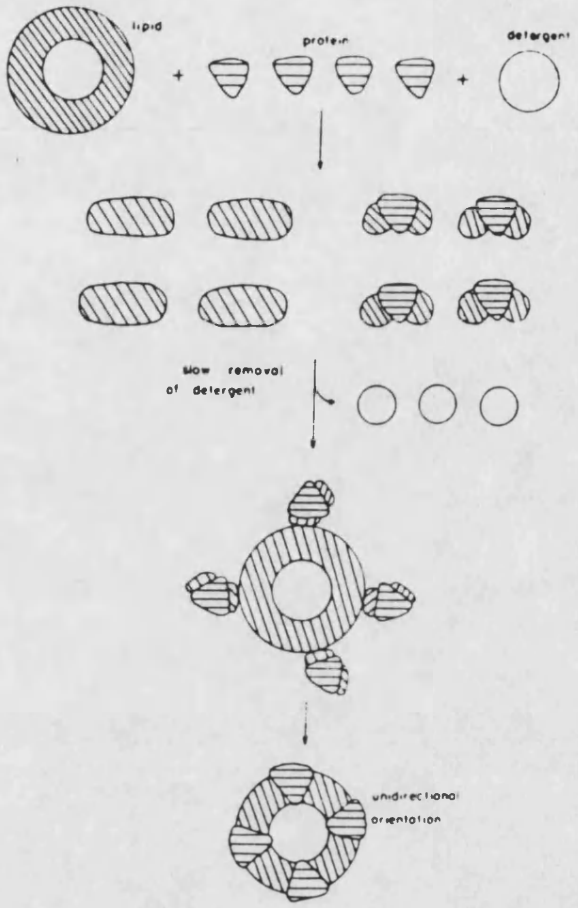


Fig. 9 A possible sequence of events leading to reconstitution by detergent dialysis. Initially mixed micelles of lipids and detergents and lipid-detergent-protein are formed. As detergent is gradually removed lipid-detergent micelles coalesce to form loose structures resembling liposomes. Further reduction in detergent causes lipid-protein micelles to adhere to the liposomes. The remaining detergent catalyses fusion of the lipids surrounding the protein with the liposome matrix resulting in asymmetric insertion of the protein into the liposome (Eytan, 1982).

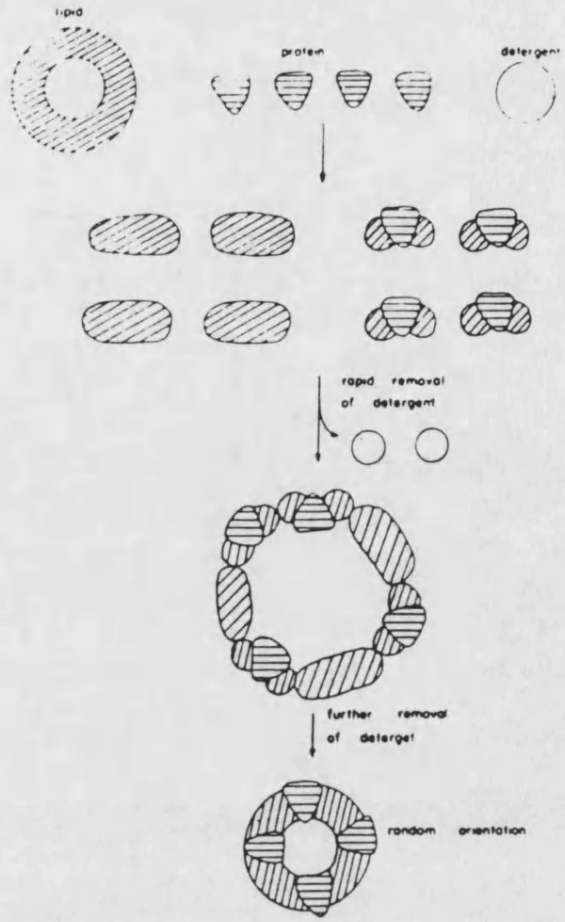


Fig. 10 The figure represents a possible scheme for reconstitution by detergent dilution. Initially the proteins, lipid and detergent form mixed micelles. Rapid removal of detergent produces a structure containing lipids and proteins, which upon reorganisation is transformed into tight proteoliposomes. Rapid detergent removal prevents the gradual building of proteoliposomes (Eytan, 1982).

the transport protein or plasma membrane particles in the presence of lipid, over long periods of time, up to 30 minutes. This time can be reduced if liposomes are wholly formed by sonication of the lipids, with subsequent addition of the transport protein, and further brief sonication, or if the liposome protein mixture is sonicated in the presence of a solvent *ie.* 1-bromohexane (Racker *et al.*, 1979).

Sonication avoids the problems of detergent inactivation, disassembly of protein complexes, and the need to remove detergents. It has however been demonstrated that some irradiation harms proteins (Carroll Racker, 1977; Banerjee *et al.*, 1977). In the case of Bacteriorhodopsin sonication proved superior to cholate dialysis (Racker, 1973), but inferior to n-octyl-B-D-glucopyranoside dilution (Racker *et al.*, 1979), and is the only method for reconstituting the Na^+K^+ATPase from the electric eel (Racker & Fisher, 1975).

An improvement in the sonication method was developed by Kasahara and Hinkle (1976) in which the D-glucose transporter of red blood cells was incorporated into liposomes by a freeze/thaw, sonication procedure. A Triton X-100 extract of red blood cell "ghosts" was treated to remove the detergent and the resulting suspension of protein and lipid added to an excess of phosphatidyl choline vesicles (prepared by sonication). Rapid freezing of the mixture and thawing at room temperature fused the natural protein:lipid micelles with the prepared vesicles. Brief sonication (20 seconds)

broke up lipid aggregates and conferred greatly improved transport activity on the proteoliposomes. To date the main problem with this technique has been its reproducibility, and possible inactivation of proteins by detergent. It has been used successfully in reconstituting the purified Na^+K^+ pumps from the electroplax of Electrophorus electricus (Hokin & Dixon, 1979), and electric eels (Racker, 1979), Bacteriorhodopsin (Racker, 1979) and the D-glucose transporter of Saccharomyces cerevisiae (Franzusoff & Cirillo , 1983a).

One of the advantages of the Kasahara and Hinkle reconstitution procedure is its application to whole or fragments of plasma membrane. Since the technique does not involve special lipids or detergents the transporter protein remains in its native environment when inserted into the artificial liposome membrane matrix. Wheeler (1986) reconstituted human erythrocyte "ghost " (erythrocytes emptied of their contents) membranes into artificial liposomes. Fanzusoff and Cirillo (1983b) and Ongjoco etal. (1987) reconstituted D-glucose transport of Saccharomyces cerevisiae by freeze/thaw sonication of yeast plasma membrane particles with preformed liposomes.

2.1.3 Direct incorporation reconstitution procedures

Membrane components can be incorporated directly into preformed liposomes without detergent; or with detergents at low concentrations, that catalyse the incorporation of the protein into the lipid bilayer. Fig. 11 demonstrates

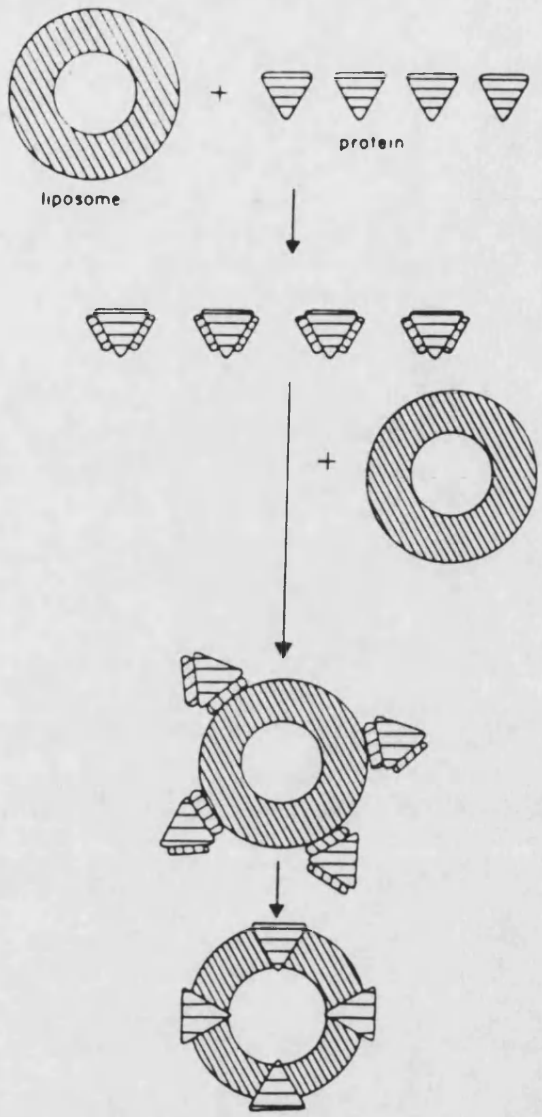


Fig. 11 A possible scheme for direct incorporation of membrane proteins into liposomes. Upon addition of liposomes to delipidated membrane proteins, part of the liposomes are broken down and lipid-protein complexes are formed, with non-vectorial functions. If an excess of liposomes are present the proteins are adsorbed to them. Fusion of the liposome lipid matrix and the lipids enveloping the proteins then occurs, provided that either low detergent concentrations, or a specific lipid composition are present. The protein is preferentially inserted with its more hydrophilic pole exposed to the exterior (Eytan, 1982).

a possible mechanism for the insertion process.

2.1.3.1 Direct incorporation reconstitution procedure with detergent

In this procedure the preformed liposomes are exposed to the transport protein in the presence of very small amounts of detergent for up to several hours, at temperatures from 0°C to 20°C. Detergents such as cholate, n-octyl-β-D-glucopyranoside and lysolecithin (formed by the action of phospholipases on phospholipids) have been used successfully.

Early attempts by Eytan et al. (1975) utilised lysolecithin because of its closer resemblance to natural lipids than detergents. Bovine heart mitochondrial cytochrome c oxidase incubated with liposomes containing 10% (w/w) lysolecithin in the total lipids required 20 hours for maximum incorporation. Most importantly the protein was asymmetrically inserted, which is not the case with most reconstitution techniques. Similar results were obtained when lysolecithin was replaced by cholate at the low concentration of 0.1% (w/v) when incubated for 20 hours.

2.1.3.2 Direct incorporation reconstitution procedure without detergents

Direct incorporation of proteins into liposomes without detergents has proved possible, if the correct mixture of phospholipids, particularly acidic phospholipids, is used in the preparation of the liposomes. Racker (1979) found that cytochrome c oxidase required 30%

phosphatidyl choline, 20-30% phosphatidyl inositol, and 10% cardiolipin for optimum incorporation . In contrast to low levels of detergent, the incorporation was rapid, requiring minutes rather than hours, and could be stimulated by magnesium ions. Temperatures lower than room temperature, EDTA and low levels of detergents such as cholate and lysolecithin slowed down or inhibited incorporation.

The most important aspect of this technique was the unidirectional orientation of the proteins, and the option of sequentially incorporating proteins into the liposomes. Miller and Racker (1976) reconstituted bacteriorhodopsin by sonication in liposomes containing acidic phospholipids to which was added oligomycin sensitive ATPase, which incorporated into the liposomes with no additional procedures. The proteoliposomes so formed catalysed the light-dependent formation of ATP. A modification of this system using 30% phosphatidyl serine or cardiolipin (but not phosphatidyl inositol) produced vesicles that fused rapidly in the presence of Ca^{2+}. This system allowed fusion of vesicles containing proteins reconstituted by different techniques, i.e. cytochrome c oxidase by cholate dialysis and the hydrophobic protein of mitochondria produced by sonication (Racker, 1979).

The most recent direct incorporation method (Dencher,1986) used the short chain lecithin diheptanoyl phosphatidyl choline (20mol % lipid) to incorporate

the purple membrane from Halobacterium halobium into long chain lecithin vesicles, *ie.* dipalmitoyl phosphatidyl choline. Incorporation required temperatures above the transition temperature of the preformed liposomes, incubation with purple membranes and 20mol % diheptanoyl phosphatidyl choline. Total incorporation of the purple membrane was achieved with dimyristoyl phosphatidyl choline, but not if dipalmitoyl phosphatidyl choline was used.

3.0 USE OF DETERGENTS IN PROTEIN EXTRACTION AND RECONSTITUTION

To study effectively membrane proteins, biological membranes need to be dissociated and the protein components reassembled to study activity and function. Detergent solubilisation represents the most useful and applicable approach. The complexity of native membranes, and the interactions of proteins, and proteins and lipids means that no one detergent can be effective for all proteins. Ideally the detergent should give maximum dissociation of the membrane whilst preserving the enzymic, antigenic, or other activity/function of the protein. Four other criteria are also desirable in an effective detergent (Hildreth, 1982).

(1) Electrical neutrality to avoid alteration of the charge properties of the solubilised protein.

(2) A high critical micelle concentration, *ie.* the concentration above which the detergent forms micelles, producing two populations of detergent, monomers and micelles. Further addition of detergent forms more micelles rather than monomers. A high critical micelle concentration (CMC) facilitates the removal by dialysis or dilution.

(3) Optical transparency in the ultra-violet region to permit spectrophotometric detection of the protein.

(4) A well-defined chemical composition and high purity to ensure reproducibility.

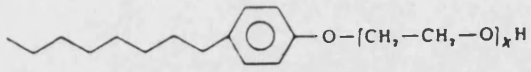
3.1 Non-ionic detergents

Some of the most widely used detergents are the neutral non-ionic polyoxyethylene ether detergents, fig. 12, which have several variations on the basic structure, two of the most important being the Brij series (alkyl polyoxyethylene ethers), fig. 12(1), with the general formula $C_nH_{2n+1}(OCH_2CH_2)_xOH$, and the Triton X series of detergents (p-tert-octylphenyl-polyoxyethylene ethers), fig. 12(3), containing a phenyl ring between the alkyl group and the polyoxyethylene head group.

Both of these detergents have good solubilising characteristics, and non-denaturing properties, particularly the Triton series of detergents; however, they absorb light at 280nm, and are poor detergents for breaking protein-protein interactions. The major drawback to their use is the large micelle size making them incompatable with gel filtration procedures (Hildreth, 1982) and the low critical micelle concentration, ie. Triton X100, critical micelle concentration 0.3mM (Helenius & Simons, 1975), precluding removal by dilution or dialysis. Removal therefore requires polystyrene beads (Hollaway, 1973). Triton X-100 has been used to solubilise the D-glucose transporter of erythrocytes by Kasahara and Hinkle (1976).

The β-D-alkyl glucosides and N-D-Gluco-N-methyl alkanamides (MEGA) compounds, fig. 12(5) and (6), represent the latest detergents introduced in the non-ionic range. Both detergents are good solubilisers, with no absorption

1



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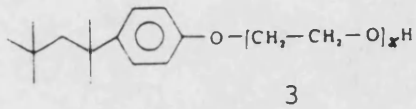

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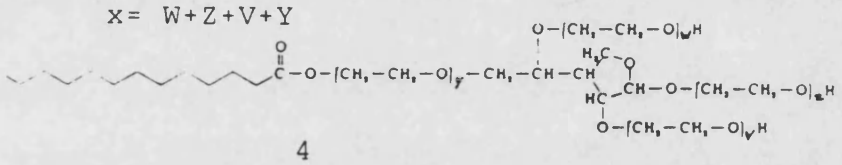

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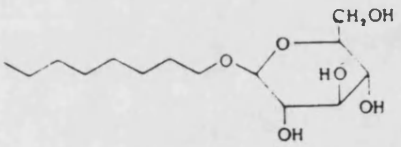

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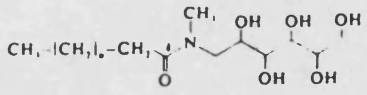


n = 5, 6 or 7

6

Fig. 12 Non ionic detergents. 1)alkyl polyoxy ethylene ethers (BRIJ series). 2) alkyl phenyl polyoxy-ethylene ethers (Triton N series). 3) p-tert-octylphenylpolyoxyethylene ethers (Triton X series and Nonident P40). 4) Polyoxyethylene sorbitol esters (Tween series). For all the above x=total number of oxyethylene units i.e. 9.6 for Triton X100. 5) n-octyl-B-D-glucopyranoside, and 6) N-D-gluco-N-methyl alkanamides.

at 280nm, but most importantly a high critical micelle concentration allowing removal by dilution or dialysis. N-octyl-β-D-glucopyranoside (8 carbon chain tail) has a critical micelle concentration of 25mM, which decreases with increasing chain length; C_{10} critical micelle concentration is 2.2mM, and C_{12} 0.19mM (Helenius et al., 1979). The critical micelle concentration of MEGA 9 and 10 where 9 and 10 refer to the number of carbons on the hydrophobic tail, have not been determined, but are believed to be similar to n-octyl-β-D-glucopyranoside (Hildreth, 1982).

N-octyl-β-D-glucopyranoside represents the most widely used detergent of the series for solubilisation and reconstitution as outlined previously, but its expense prevents large scale use. The MEGA series of detergents have not been widely used, but have been successful in solubilising and reconstituting antigenic determinants from B lymphoblast cells. MEGA 9 (and 10) released protein as efficiently as n-octyl-β-D-glucopyranoside, sodium cholate and Nonident P40 (at 0.5% w/v). MEGA 10 proved more efficient at releasing protein than other non-ionic detergents including Nonident P40, Triton X-100 and Brij 96 (Hildreth, 1982). To date the main problem with MEGA 10 is its insolubility at low temperatures ie. 4°C.

3.2 ZWITTERIONIC DETERGENTS

Zwitterionic detergents are detergents with a hydrophobic tail and a head group containing a negative

and positive charge. Lysophospholipids, fig. 13(1), represent the most lipid-like detergents, with powerful solubilising power. Navarette and Serrano (1983) compared the solubilising power and retention of ATPase activity in detergent extracts of yeast plasma membrane and mitochondria. In the plasma membrane, egg lysolecithin solubilised up to 75% of the total membrane protein with retention of ATPase activity. Taurodeoxycholate, Triton X-100 and n-octyl-β-D-glucopyranoside released no more than 30% of the plasma membrane protein, but released the same amount of ATPase activity.

Egg lysolecithin has a critical micelle concentration of 20 to 200μmolar.(Helenius et al., 1979) and a large critical micelle size. These properties make the detergent extremely difficult to remove from the detergent extracts, however it has been used in protein purification and reconstitution systems.

Serrano (1984) successfully purified the plasma membrane ATPase from oat root and reconstituted it into liposomes using a freeze-thaw technique.

More recently synthetic zwitterionic detergents have been synthesised, most notably the sulphobetaines, fig. 13(2) (Gonenne & Ernst, 1978), containing a tertiary amine and sulphate head group (and hence overall neutrality) connected to a variable length alkyl hydrophobic tail. The overall neutrality of the head group is stable over a wide pH range.

In their general properties the sulphobetaines resemble the non-ionic detergents, with a critical micelle

concentration altered little by ionic strength, but decreasing logarithmically with increasing tail length. They also avoid the problems of absorbance at 280nm.

Using 3T6 mouse fibroblasts Gonenne and Ernst (1978), utilising sulphobetaine detergents, found that increasing tail chain length resulted in increasing solubilising power (n=8 to 16 total chain length) with a maximum reached at C_{14}. Above this value the detergent precipitated from solution at 4°C. At C_8 and C_{10} tail chain length 5'-nucleotidase was activated but not released, and with C_{12} to C_{18} 5'-nucleotidase was activated and released, results unobtainable using Triton X-100.

In the solubilisation of the yeast plasma membrane, Navarrete and Serrano(1983) found similar properties. Plasma membrane protein and ATPase were most effectively extracted with Zwittergent 14 and Zwittergent 16 (Zwittergent is the common name for sulphobetaine detergents), far in excess of that released by taurodeoxycholate, n-octyl-β-D-glucopyranoside and Triton X-100, and comparable with that released by lysolecithin. Zwittergents have been demonstrated to denature proteins, eg. Zwittergent 14 on cytochrome P450 (Hjelmeland et al., 1979) and an oat root ATPase (Vara & Serrano, 1982).

3.3 BILE SALT DETERGENTS

Bile salts such as cholate, fig. 13(3), differ from the detergents so far mentioned in that they are a rigid steroid structure with hydroxyl groups (3 for cholate,

2 for deoxycholate) arranged on one surface of the molecule, producing a hydrophilic and hydrophobic face. There is no hydrophilic head or hydrophobic tail.

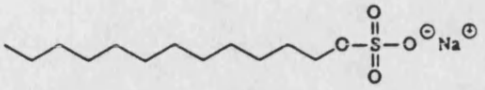
The bile salts are optically clear at 280nm with small micelle sizes and high critical micelle concentration; sodium cholate, approximately 10mM, sodium glycocholate, 13mM and other derivatives with critical micelle concentrations of 1 to 3mM (Helenius et al., 1979).

The effectiveness of all the bile salts is susceptible to ionic strength which alters the critical micelle concentration. Lowering the pH leads to precipitation of the detergent (pH6.5 for sodium cholate, pH 6.9 for sodium deoxycholate; Helenius & Simons, 1975). Counterions such as Ca^{2+} and Mg^{2+} precipitate cholate and deoxycholate and total bile salt concentration affects the CMC, since both monomer and micelle forms increase with concentration.

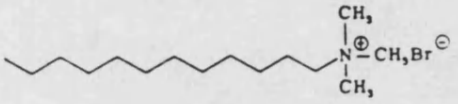
Bile salts, particularly cholate, have proved very useful detergents in protein extraction and reconstitution eg. in the reconstitution of the Ca^{2+} pump of sarcoplasmic reticulum by Racker et al.(1975). They have the ability to dissociate some protein interactions, unlike the alkyl polyoxyether detergents, and are easily removed by dialysis or dilution.

3.4 ALKYL IONIC DETERGENTS

These detergents such as sodium dodecyl sulphate and cetyl-trimethyl ammonium bromide, fig. 14, 1 and 2



(1)



(2)

Fig. 14 Alkyl ionic detergents.

1. Sodium dodecyl sulphate
2. Cetyl trimethyl ammonium bromide

respectively, completely solubilise membranes, usually dissociating complex proteins into constituent polypeptide chains resulting in denaturation of the protein (Helenius et al., 1979). They are never the choice detergent for reconstitution; however, mycoplasma membranes solubilised by sodium dodecyl sulphate, spontaneously reaggregated to form vesicles with a structure similar to the original mycoplasma membrane, provided that a divalent metal ion was present (Rottem et al., 1968).

4.0 OCCURENCE AND FUNCTION OF DIHYDROLIPOAMIDE DEHYDROGENASE

Dihydrolipoamide dehydrogenase is an integral component of the pyruvate, 2-oxoglutarate and branched 2-oxoacid dehydrogenase complexes (Reed, 1974; Perham, 1975; Pettit et al., 1978) and of the glycine enzyme cleavage system (Kikuchi & Hiraga , 1982). Apart from the above, recent evidence suggests that the enzyme has another role specific to a location in the plasma membrane of the cell with as yet undefined function.

Dihydrolipoamide dehydrogenase catalyses the NAD^+-dependent oxidation of dihydrolipoamide (Williams, 1976), involving the alternate oxidation and reduction of an intrachain disulphide bond, and a base (B) on the enzyme, see fig. 15. The enzyme's role in association with the 2-oxoacid dehydrogenase complexes has only been found in aerobic respiratory organisms. Its role cannot be associated with 2-oxoacid dehydrogenase complexes in archaebacteria because they lack this enzyme complex. Kerscher & Oesterhelt (1982) suggested that the association with 2-oxoacid dehydrogenase complexes evolved after the development of oxidative phosphorylation, taking dihydrolipoamide dehydrogenase from another role in the cell.

Archaebacteria possessing no 2-oxoacid dehydrogenase complexes convert their pyruvate and 2-oxoglutarate to the corresponding acylCoA thioesters using oxidoreductases containing no lipoic acid (Kerscher &

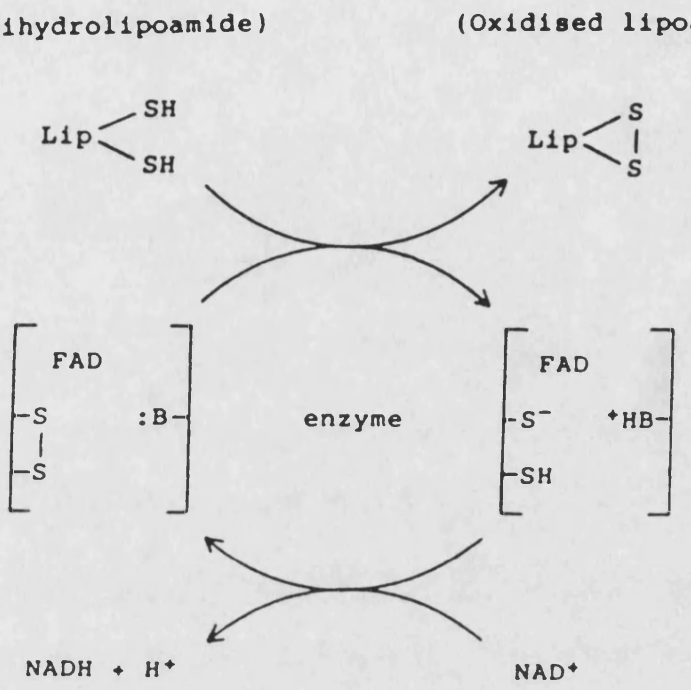


Fig. 15 Enzymic action of dihydrolipoamide dehydrogenase. Oxidation of dihydrolipoamide results in reduction of an intrachain disulphide bond and a base (B). The disulphide bond is then reformed and the base deprotonated during the production of NADH from NAD^+.

Oesterhelt,1982). Dihydrolipoamide dehydrogenase has however been found in the archaebacteria (Danson et al., 1984; Smith et al., 1987; Danson, 1988).The purified enzyme from H. halobium exhibits alternate oxidation and reduction of disulphide bonds (Danson et al., 1986) and sensitivity to dithiol specific reagents, displaying similar properties to the dihydrolipoamide dehydrogenases from eubacteria and eukaryotes.(Danson, unpublished data). More importantly the enzyme from Thermoplasma acidophilum appears to be associated with the plasma membrane.

Respiratory eubacteria contain dihydrolipoamide dehydrogenase in several multienzyme complexes, but not exclusively so. Owen et al.(1980) demonstrated that in Escherichia coli the enzyme fractionates to similar extents between membrane and cytoplasm, with a membrane vesicle system that suggested dihydrolipoamide dehydrogenase was responsible for the NADH-dependent transport of amino acids.

E. coli deficient in the synthesis of lipoic acid grow in the presence of acetate and succinate, with functional lactose permease and PEP-glucose phosphotransferase, but with greatly reduced binding-protein dependent transport of ribose, galactose and maltose (Richarme, 1985). When lipoic acid is supplied in the growth media, the lipoic acid dependent transports are completely inhibited by the dithiol-specific arsenate. Richarme and Heine (1986) demonstrated that dihydrolipoamide dehydrogenase activity can be detected in an E. coli

strain deleted for the lpd gene which codes for the enzyme in the 2-oxoacid dehydrogenase complexes. Residual activity was 15% of that in the parental strain and was stimulated by galactose and maltose. No stimulation was observed in mutant deficient in the corresponding binding-protein dependent transport system. This evidence suggests that lipoic acid and dihydrolipoamide dehydrogenase are connected with these sugar transport systems.

Dihydrolipoamide dehydrogenase has recently been found in Eukaryotic cells in the plasma membrane fraction of rat adipocytes (A.R. Karim, G.D. Holman and M.J. Danson, unpublished data). Mitochondrial contamination as determined by succinate dehydrogenase activity and plasma membranes by the marker 5'-nucleotidase, produced a succinate dehydrogenase to dihydrolipoamide dehydrogenase ratio in the plasma membranes, one fifth that found in the mitochondrial fraction. This suggested dihydro-lipoamide dehydrogenase activity specific to the plasma membranes.

MATERIALS AND METHODS

1.0 INFECTION AND ISOLATION OF T.B. BRUCEI

Materials

Potassium dihydrogen orthophosphate, magnesium sulphate, potassium chloride and D-glucose were analar grade from BDH Chemicals Ltd. Sodium chloride was analar grade from Fisons PLC and T.b. brucei were strain MITat 1.1 (supplied by Dr. H.P. Voorheis, Trinity College, Dublin). Adult male or female, Wistar and/or Sprague-Dawley rats of weight 200-800 grams, were supplied by the University of Bath animal house. Krebs Ringer phosphate buffer (KRB) consisted of potassium dihydrogen orthophosphate (22mM), sodium chloride (98mM), potassium chloride (2mM) and magnesium sulphate (1mM), made up in double distilled water, taken to pH8.0 with 10M sodium hydroxide. Heparin (Sigma, grade I, porcine intestinal mucosa) was made up at 200 units per millilitre with Krebs Ringer phosphate buffer.

Whatman pre-swollen DE52 diethylaminoethyl cellulose anion exchanger was equilibrated in KRB (15 to 30ml of KRB per gram of DE52) and taken to pH8.0 with the addition of 10M sodium hydroxide. "Fines" above the slurry were removed by aspiration, and the "slurry" washed thrice with KRB. After each wash "Fines" were removed by aspiration, and the resulting DE52 "slurry" stored at 4°C until required. SABRE sterile hypodermic needles, 0.8mm x 40mm, were used for infection and bleeding into Gillette 20ml plastic disposable syringes. Diethyl ether was supplied by James Burrough, England.

Method

T.b. brucei (stored in whole rat blood, 12%(w/v) glycerol in heparinised capillary tubes, at -196°C), were taken up in Krebs Ringer phosphate buffer, pH8.0, 10mM D-glucose at 4°C. Adult rats were innoculated with 1 to 3 x 10^7 long slender blood stream form T.b. brucei by intraperitoneal injection (Eisenthal & Panes, 1985), with total innoculation volume 0.5 to 0.75ml per rat.

Seventy-one hours post-infection the rats were anaesthetised under ether and the abdominal cavity cut open. The rats were bled of 8 to 20ml of blood into a 20ml syringe containing 1 to 2ml of heparin solution by insertion of the hypodermic needle into the bifurcation of the common iliac artery.

T.b. brucei were removed from the rat blood components by centrifugation of whole blood at 1000g, 4°C, in an IEC CENTRA-3R refrigerated centrifuge. Blood plasma was removed by aspiration and the trypanosomes overlying the packed erythrocytes gently aspirated into Krebs Ringer phosphate buffer, pH8.0, 10mM D-glucose, 4°C, and removed from the erythrocytes. The trypanosomes were washed once with Krebs Ringer phosphate buffer, pH8.0, 10mM D-glucose, and resuspended to approximately 10ml with KRB, 10mM D-glucose.

Platelets, residual red blood cells and white blood cells were separated from the trypanosomes by passage through a DEAE 52 cellulose column (5 to 10ml packed volume), equilibrated with KRB, 10mM D-glucose, at 4°C. The cells were washed once with KRB, 10mM D-glucose

and resuspended in the same buffer to approximately 1×10^9 cells/ml at 4°C and kept at 4°C until required.

Cell counts were determined on a Gallenkamp haemocytometer.

2.0 T.B. BRUCEI PLASMA MEMBRANE ISOLATION AND PURIFICATION

The process principally follows that developed by Voorheis et al. (1979) involving the osmotic swelling of the trypanosomes and disruption by the mechanical shear force developed in a tight fitting Dounce type homogeniser.

Materials

Tes (N-tris[hydroxymethyl]methyl-2 -aminoethane sulphonic acid), EDTA (disodium ethylenediaminetetra-acetic acid), PMSF (phenyl methylsulphonylfluoride) and leupeptin (synthetic hemisulphate) were supplied by Sigma Chemical Company. Analar 2-mercaptoethanol, potassium chloride, magnesium chloride and potassium dihydrogen orthophosphate were supplied by B.D.H. Chemicals Ltd., and sucrose from Fisons PLC. Deoxyribonucleotidase class I was purchased from Cooper Biomedical supplies. Tes buffer (Tes/EDTA) consisted of Tes (2mM), potassium chloride (150mM), EDTA (1mM), 2-mercaptoethanol (1mM), PMSF (0.1mM) dissolved in double distilled water taken to pH7.5 with 1M sodium hydroxide. "Swelling" buffer consisted of Tes (1mM), 2-mercaptoethanol (1mM), PMSF (0.1mM) and leupeptin (5µg/ml). Leupeptin was dissolved in methanol and added to buffers with vigorous stirring, and PMSF (in 100µl of dimethyl sulphoxide) added to warmed buffers with vigorous stirring. DNAase treatment buffer (Tes/$MgCl_2$) was the same as Tes/EDTA, except EDTA was replaced with magnesium chloride (5mM).

Analar sucrose was supplied by Fisons PLC. A stock solution of 80% (w/v) sucrose in Tes/EDTA, was diluted with Tes/EDTA, 0.2mM PMSF to give 5 x 5ml concentrations of sucrose and 60% (twice), 55%, 50% and 45% (w/v) and 6ml of 40% (w/v) sucrose. The 40% to 60% sucrose gradients were produced by gently pipetting the 5 x 5ml concentrations of sucrose (60% first) into Beckman nitrocellulose ultracentrifuge tubes, topped with 2ml of 40% (w/v) sucrose. The sucrose grdient was subsequently refined by replacing one of the 60% (w/v) sucrose layers with a 52% (w/v) sucrose layer, such that the 5 x 5ml sucrose layers were 60%, 55%, 52%, 50% and 45% (w/v) sucrose, topped with 2ml of 40% (w/v) sucrose. A continuous gradient was formed by standing the tubes in ice for 3 hours.

In later experiments the buffer system was changed to phosphate buffered saline (PBS), sodium dihydrogen orthophosphate (12.5mM), sodium chloride (150mM), and PMSF (0.1mM). Tes/EDTA was replaced by PBS, 0.1mM EDTA pH7.4, Tes/$MgCl_2$ by PBS, 5mM $MgCl_2$, and swelling buffer by double distilled water, 0.1mM PMSF, 5µg/ml leupeptin.

Methods

T.b. brucei in KRB, 10mM D-glucose, 1-4 x 10^{10} cells were spun down at 1000g, 10min, 4°C.

Cell pellet

↓

(1) Cells suspended in 10ml Tes/EDTA, 4°C.

(2) Up to 30ml of swelling buffer added to the cells with stirring, to give swollen cells, with little or no flagellar movement.

(continued overleaf)

(3) Cell suspension homogenised by one stroke of a tight fitting Dounce homogeniser.

(4) 2ml of 3M KCl added for each 40ml of homogenate

(5) Homogenate centrifuged 7500g, 10 sec (8x50ml rotor, MSE 18 centrifuge), 4°C.

Supernatant S1 discarded

Pellet P1

Membranes resuspended in 50ml Tes/EDTA, 5µg/ml leupeptin and collected by centrifugation 7500g, 10sec, 4°C (8x50ml rotor, MSE 18 centrifuge).

Supernatant S2 discarded

Pellet P2

(1) suspended in 10ml Tes/$MgCl_2$ 20°C.

(2) 227u DNAase added, stood 5min, 20°C.

(3) DNAase action was ended by addition of 40ml Tes/EDTA 4°C.

(4) Plasma membranes collected by centrifugation 7500g, 10sec, 4°C (8x50ml rotor, MSE 18 centrifuge).

Supernatant S3 discarded

Pellet P3

(1) suspended in 4-8ml 40% (w/v) sucrose at 4°C

(2) layered on 40-60% sucrose gradient

(3) centrifuged 70000g, 3h, 4°C (SW 28 rotor, in Beckman L65 ultracentrifuge)

Rest of gradient discarded

Prominant dense band at 51.3% (w/v) sucrose

(1) diluted with 50ml Tes/EDTA 4°C.

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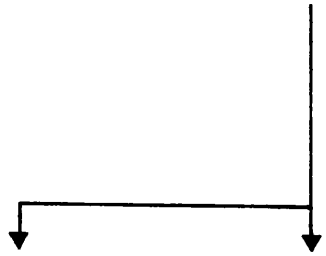


(2) plasma membranes collected by centrifugation 7,500g, 4°C, 2min. (8x50ml rotor, MSE 18 centrifuge)

(3) 1 and 2 repeated twice

Supernatant S4 discarded

Plasma membrane pellet P4 used fresh for experiments, or rapidly frozen in liquid nitrogen and stored at -90°C until required.

3.0 MARKER ENZYME ASSAYS TO DETERMINE PLASMA MEMBRANE PURITY

Plasma membranes and various fractions taken from the plasma membrane isolation and purification procedure, were assayed for organelle marker enzymes to determine plasma membrane purity. The enzymes assayed were, hexokinase, L-glycerol-3-phosphate dehydrogenase, D-glucose-6-phosphatase, pyruvate kinase, D-glucose-6-phosphate isomerase, malate dehydrogenase, α-glucosidase (suggested by Opperdoes et al., 1976, to be a plasma membrane marker enzyme), myokinase (adenylate kinase), ouabain sensitive Na^+K^+ATPase, oligomycin sensitive $Na^+K^+Mg^{2+}$ stimulated ATPase and the sn-glycerol-3-phosphate oxidase.

Materials

Disodium adenosine triphosphate (ATP, equine muscle), dihydroxyacetone phosphate, imidazole, glycylglycine, ascorbic acid, D-fructose-6-phosphate (F-6-P), oxaloacetate, D,L-dithiothreitol, adenosine monophosphate (A-5-MP, acid form Type I from yeast), Fiske and Subbarow reducer (1-amino-2-napthol-4-sulphonic acid, 0.8% sodium sulphite and sodium bisulphite, 5g/31.5ml double distilled water), acidmolybdate (ammonium molybdate. $4H_20$, 1.25g/l in 2.5N sulphuric acid), L-α-glycerophosphate (di[monocyclohexylammonium] salt), D-glucose-6-phosphate (G-6-P), ouabain (strophanthin G octahydrate), oligomycin, pyruvate kinase (rabbit muscle type II 465u/mg protein,

4.3mg protein/ml), D-glucose-6-phosphate dehydrogenase (leuconostoc mesenteroides Type XXIII $NADP^+$dependent, 275-400u/mg protein), were all supplied by Sigma Chemical Co. (Poole, Dorset).

Phosphoenol pyruvate (PEP, monohexylamine salt), disodium adenosine diphosphate (ADP), analar trisodium citrate, trichloroacetic acid and maltose were purchased from BDH Chemical Co.

Nicotinamide adenine dinucleotide phosphate (oxidised form, $NADP^+$), nicotinamide adenine dinucleotide (oxidised and reduced forms, NAD^+ and NADH respectively), hexokinase (140u/mg protein, 10mg protein/ml), and lactate dehydrogenase (pig heart, 300u/mg protein, 5mg protein/ml) were supplied by Boehringer Mannheim, Germany.

Analar sucrose, triethanolamine hydrochloride (TEA), sodium arsenite and ammonium molybdate were purchased from Fisons PLC., and Ultrapure Triton X-100 from Pierce Chemical Co., U.S.A. Dihydrolipoamide was a gift from Dr. M.J. Danson, Bath University, Bath, United Kingdom.

All absorbance measurements were carried out on a Phillips Unicam SP6-450 spectrophotometer, or Cecil Œ 212 ultra-violet spectrophotometer and recorded on a Rikadenki chart recorder.

Methods

All the enzymes were assayed at 37°C, pH7.6, in the presence and absence of 0.1% (w/v) Triton X-100 and

initiated by addition of sample to the reagents at the concentrations below, unless otherwise stated. All samples were assayed for background and endogenous rates, which were subtracted where necessary.

Hexokinase (Bergemyer , 1974a)

In the presence of D-glucose and ATP, hexokinase produced D-glucose-6-phosphate which was then oxidised by D-glucose-6-phosphate dehydrogenase, concurrently $NADP^+$ was reduced to NADPH, allowing the reaction to be followed by the increase in absorbance at 340nm.

$$\text{D-glucose} + \text{ATP} \xrightarrow[\text{hexokinase}]{} \text{D-glucose-6-phosphate} + \text{ADP}$$

$$\text{D-glucose-6-phosphate} + NADP^+ \xrightarrow[\text{dehydrogenase}]{\text{glucose-6-phosphate}} \text{NADPH} + H^+ + \text{6-phosphogluconolactone}$$

$$\text{6-phosphogluconolactone} + H_2O \xrightarrow[\text{½ life 20min}]{\text{non-enzymatic}} \text{6-phosphogluconate}$$

The assay contained NADP (0.13mM), D-glucose (111mM), ATP (0.5mM), magnesium chloride (5mM), D-glucose-6-phosphate dehydrogenase (10µg/ml), TEA (50mM, pH7.6), and 5 to 50µl of sample, in a total volume of 1ml.

L-glycerol-3-phosphate dehydrogenase (Panes, 1988)

Dihydroxyacetone phosphate was reduced by the activity of G-3-P dehydrogenase in the presence of NADH producing α-glycerol phosphate and NAD^+. The loss in absorbance at 340nm allows the reaction to be followed.

$$DHAP + NADH + H^{+} \xrightarrow[\text{dehydrogenase}]{\text{L-glycerol-3-phosphate}} \alpha\text{-glycerol phosphate} + NAD^{+}$$

Assay reagents were NADH (0.14mM), dihydroxyacetone phosphate (1mM), TEA (50mM, pH7.6) and sample, 5 to 50µl in a total volume of 1ml.

Glucose-6-phosphatase (Bergemyer, 1974b)

Inorganic phosphate was released from D-glucose-6-phosphate by the action of the phosphatase, and was measured colourimetrically by addition of molybdate. This yields a molybdate-phosphate complex producing a blue colour upon reduction. Excess molybdate was bound by citrate/arsenite solution, preventing interference from background inorganic phosphate released from ATP under the acidic conditions.

$$\text{D-glucose-6-phosphate} \xrightarrow[\text{phosphatase}]{\text{Glucose-6-}} \text{D-glucose} + Pi$$

The assay was carried out in sucrose (62.5mM), EDTA (0.25mM), D-glucose-6-phosphate (25mM), imidazole/glycylglycine (50mM/50mM, pH7.0), with 50µl of sample to start the reaction, in a total volume of 200µl. Incubation was at 37°C, for 10min, and the assay stopped by the addition of 1ml of ascorbic acid/trichloro-acetic acid (2% (w/v) & 10% (w/v) respectively). Precipitated protein and lipid were removed by centrifugation at 10,000g, 5min, and 500µl of the supernatant added to 250µl of ammonium molybdate solution (1% (w/v)). The tube

was mixed well and 500µl of arsenite/citrate solution (2% (w/v) respectively) added to stabilise the colour. Absorbance at 700nm was read after 10min.

Pyruvate kinase (Bergemyer, 1974c)

Pyruvate released by the activity of pyruvate kinase was reduced to lactate by lactate dehydrogenase, whilst NADH was oxidised to NAD^+. The reaction was followed by the loss in absorbance at 340nm.

$$\text{Phosphoenolpyruvate} + \text{ADP} \xrightarrow[\text{kinase}]{\text{pyruvate}} \text{pyruvate} + \text{ATP}$$

$$\text{pyruvate} + \text{NADH} + H^+ \xrightarrow[\text{dehydrogenase}]{\text{lactate}} \text{lactate} + NAD^+$$

The assay contained PEP (0.5mM), TEA (50mM, pH7.6), ADP (5mM), potassium chloride (100mM), magnesium chloride (5mM), NADH (0.14mM), lactate dehydrogenase (50µg/ml), and 5 to 25µl of sample in a total volume of 1ml.

D-glucose-6-phosphate isomerase

D-fructose-6-phosphate was converted to D-glucose-6-phosphate by the isomerase, which was then oxidised by glucose-6-phosphate dehydrogenase reducing $NADP^+$ to NADPH. The reaction was followed by the increase in absorbance at 340nm.

$$\text{D-fructose-6-phosphate} \xrightarrow[\text{isomerase}]{\text{glucose-6-phosphate}} \text{D-glucose-6-phosphate}$$

$$\text{D-glucose-6-phosphate} + NADP^+ \xrightarrow[\text{dehydrogenase}]{\text{glucose-6-phosphate}} \text{NADPH} + H^+ + \text{6-phosphogluconolactone}$$

Final reagent concentrations were D-fructose-6-phosphate (10mM), NADP (0.13mM), D-glucose-6-phosphate dehydrogenase (10µg/ml), TEA (50mM, pH7.6) and 5 to 20µl of sample in 1ml.

Malate dehydrogenase (Danson et al., 1987)

Malate dehydrogenase was assayed by the reduction of oxaloacetate to malate, with the concurrent oxidation of NADH to NAD^+. The reaction was followed by the decrease in absorbance at 340nm of oxidised NADH.

$$\text{oxaloacetate} + \text{NADH} + H^+ \xrightarrow[\text{dehydrogenase}]{\text{malate}} \text{malate} + NAD^+$$

The assay was carried out in potassium phosphate buffer (50mM, pH7.0), EDTA (2mM), NADH (0.1mM), and oxaloacetate (0.2mM), in a total volume of 1ml, at 30°C.

α-glucosidase (Bergemyer, 1974d)

α-Glucosidase was monitored by the cleavage of the α-1,4 glucose linkages in maltose producing two D-glucose molecules. D-glucose was assayed in the presence of ATP, hexokinase, glucose-6-phosphate dehydrogenase and NAD^+ and determined by the increase in absorbance at 340nm.

$$\text{Maltose} + H_2O \xrightarrow{\alpha\text{-glucosidase}} 2\ \text{D-glucose}$$

The enzyme was assayed at 20°C, for 24 hours, in the presence of potassium phosphate buffer (100mM, pH7.0), dithiothreitol (1mM), maltose (22.5mM), Triton X-100 (0.1% (w/v) with 200µl of sample in a total volume of 1ml. Thirty microlitre aliquots were assayed for D-glucose in potassium phosphate buffer (100mM, pH7.0),

ATP (0.5mM), $NADP^+$ (0.47mM), magnesium chloride (3.5mM), and 10µg/ml of hexokinase and glucose-6-phosphate dehydrogenase, total volume 1ml.

Myokinase (Adenylate kinase)

Two techniques were used to measure the enzyme activity, the production of ADP from ATP and A-5-MP and the production of ATP and A-5-MP from 2ADP.

i) Production of ADP from A-5-MP and ATP (Bergemyer,1974e)

The scheme for monitoring the reaction is shown below. Myokinase catalyses the production of 2ADP from 1ATP and 1A-5-MP, which was then acted upon by pyruvate kinase in the presence of PEP to produce ATP and 2 pyruvate. Lactate dehydrogenase reduced the pyruvate to lactate, whilst oxidising NADH to NAD^+. The subsequent decrease in absorbance at 340nm with the loss of NADH allowed the reaction and hence myokinase activity to be monitored.

$$\text{A-5-MP} + \text{ATP} \xrightarrow{\text{myokinase}} 2\text{ADP}$$

$$2\text{ADP} + 2\text{PEP} \xrightarrow[\text{kinase}]{\text{pyruvate}} 2\text{ATP} + 2 \text{ pyruvate}$$

$$2H^+ + 2\text{NADH} + 2 \text{ pyruvate} \xrightarrow[\text{dehydrogenase}]{\text{lactate}} 2 \text{ L-lactate} + 2NAD^+$$

The assay was carried out in a total volume of 1ml at 20°C containing sodium phosphate (50mM), EDTA (1mM) pH7.6, A-5-MP (1mM), ATP (1mM), PEP (1mM), magnesium chloride (1mM), potassium chloride (80mM), NADH (0.14mM), Triton X-100 (0.1% (w/v)) pyruvate kinase (5µl, 10 units), lactate dehydrogenase (10µl, 15 units) and 10 to 100µl of plasma membranes.

ii) Production of ATP and A-5-MP from ADP

This assay was an adaption of the hexokinase/ glucose-6-phosphate dehydrogenase assay (see α-glucosidase assays) in which ATP for the phosphorylation of D-glucose was provided by myokinase action on ADP.

$$2ADP \xrightarrow{\text{myokinase}} ATP + A\text{-}5\text{-}MP$$

$$\text{D-glucose} + ATP \xrightarrow{\text{hexokinase}} \text{D-glucose-6-phosphate} + ADP$$

$$\text{D-glucose-6-phosphate} + NADP^{+} \xrightarrow[\text{dehydrogenase}]{\text{glucose-6-phosphate}} \text{6-phosphono-glucolactone} + NADPH + H^{+}$$

The enzyme was assayed at 20°C, in a total volume of 1ml containing sodium phosphate buffer (50mM), EDTA (1mM) pH7.6, Triton X-100 (0.1%(w/v)) ADP (1.0mM), NADP (0.47mM), magnesium chloride (3.5mM), D-glucose (111mM), hexokinase (10μg/ml), and D-glucose-6-phosphate dehydrogenase (10μg/ml).

Ouabain-sensitive Na^{+} K^{+} ATPase and oligomycin-sensitive Na^{+} $K^{+}Mg^{2+}$ stimulated ATPase

The activities of these ATPases were measured using the method of Post and Sen (1967), following the release of inorganic phosphate (Pi) from ATP. Total Pi was measured by its reaction with molybdate to form phosphomolybdate which was reduced to a phosphomolybdenum blue complex by a mixture of sodium bisulphite, sodium sulphite and 1-amino-2-napthol-4-sulfonic acid according to the method of Fiske and Subbarow (1925).

Ouabain-sensitive plasma membrane ATPase was

assayed in glycylglycine/imidazole buffer (20mM respectively pH7.0), ATP (3mM), EDTA (0.1mM) in a total volume of 1.5ml. Enzyme activity was taken as the release of Pi in the presence of NaCl (100mM) and KCl (20mM), and the release of Pi in the presence of 0.17mM Ouabain with no NaCl or KCl at 37°C for 20 min with gentle agitation. For experiments where ATPase activity was required in fractions taken from the homogenate through to the final plasma membrane pellet, choline replaced sodium and potassium in the Tes/EDTA/$MgCl_2$ buffers, and EGTA replaced EDTA, to avoid the sodium in the EDTA.

Ouabain was bound to the plasma membrane ATPase by preincubation with the membranes for 20 minutes, 37°C as suggested by Voorheis (personal communication).

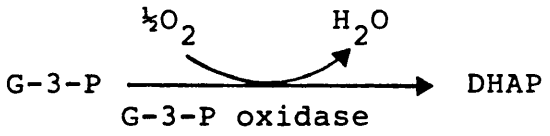
Oligomycin-sensitive ATPase was assayed under the same conditions as the plasma membrane ATPase, except sodium and potassium (100mM and 20mM respectively) were always present, and the release of inorganic phosphate was measured in the presence and absence of oligomycin (0.67µg/ml). Assays were started by the addition of ATP, with total protein of 200µg to 1mg.

Enzyme activity was halted by addition of trichloroacetic acid (final concentration 10%(w/v)). Precipitated protein and phospholipid was removed by centrifugation at 20,000g, 30 seconds, room temperature, and to 1ml of the supernatant added acid molybdate solution (final concentration 0.15%(w/v) and Fiske and Subbarow solution (final concentration 0.04%(w/v). The tubes were mixed gently and allowed to stand for 10min,

and the absorbance at 660nm read. Total inorganic phosphate was determined from a standard curve of potassium phosphate (monobasic) treated as above.

Sn-glycerol-3-phosphate oxidase

Sn-glycerol-3-phosphate oxidase was measured by the reduction in oxygen concentration (in an oxygen electrode) during the oxidation of glycerol-3-phosphate to dihydroxy acetone phosphate by the oxidase.



The assay was carried out in a Rank type oxygen electrode at 20°C, total volume 2ml in sodium phosphate (50mM) pH7.4, EDTA (0.1mM), PMSF (0.1mM) and L-α-glycerophosphate (5mM). The assay was started by addition of 10 to 100µl of sample.

3.1 DIHYDROLIPOAMIDE DEHYDROGENASE ASSAY PROTOCOL

Method

Fractions taken from stages during the purification of the trypanosome plasma membranes were stored on ice until tested for dihydrolipoamide dehydrogenase activity. Fractions assayed were the pellet P1, the final plasma membrane pellet P4, and the homogenate and supernatant S1 (both taken to 2mM Tes pH7.5, with 100mM Tes pH7.5, to increase the buffering capacity).

The enzyme was assayed by following the oxidation

of dihydrolipoamide to lipoamide, with the concurrent reduction of NAD^+ to NADH, resulting in an increase in absorbance at 340nm.

$$\text{dihydrolipoamide} + NAD^+ \xrightarrow[\text{dehydrogenase}]{\text{dihydrolipoamide}} \text{lipoamide} + NADH + H^+$$

The assay took place in 1ml total volume at 30°C, containing potassium phosphate (50mM) pH7.0, EDTA (2mM), dihydrolipoamide (0.14mM), and NAD^+ (1mM). The assay was started by the addition of enzyme, normally 5 to 100µl depending upon the fraction tested.

3.2 HEXOKINASE PHOSPHATE DONOR SPECIFICITY ASSAY

Materials

Potassium pyrophosphate was supplied by Sigma Chemical Co., and D-[U-C^{14}] glucose (1mCi/ml, 280mCi/mmol) was supplied by Amersham International Ltd., U.K. Butan-1-ol (SLR), Ammonia (SLR, 0.88 specific gravity) were purchased from Fisons PLC. Glacial acetic acid (GPR) was supplied by BDH Chemical Co., and acetone and ethanol by BP Chemicals and James Burroughs, England, respectively. Thin layer chromatography plates, Kieselgel 60F' 2.4 were purchased from Merck, Darmstadt, Germany. Polyethylene standard size scintillation vials were supplied by United Technologies, Packard, and scintillant used contained Toluene (70%) : Triton X-100 (30%) : 2,5 diphenyloxazole (PPO, 5g/l).

Method

Plasma membranes (400µl) in Tes/EDTA buffer and supernatant S1 (taken to 2mM Tes with 100mM Tes pH7.5), from the plasma membrane isolation and purification process were incubated in Tes buffer (50mM,pH7.5), 10mM phosphate donor (ATP, ADP, PEP, inorganic phosphate (Pi) and pyrophosphate (PPi), magnesium chloride (5mM), and radiolabelled D-glucose (1mM, 0.6µCi), in a total volume of 500µl. The assay was initiated by addition of D-glucose and incubated for 2.5minutes at 37°C. The assay was terminated by aliquotting 300µl of sample into 700µl of absolute ethanol.

Precipitated protein was removed by centrifugation in an OLE DICH microcentrifuge, 20,000g, 5min, 20°C. Five microlitres of supernatant was applied to a Kieselgel T.L.C. plate and run in a solvent system containing 35ml butan-1-ol, 10ml acetic acid, 5.0ml 10%(v/v) ammonia, 20ml double distilled water and 25ml acetone, in a total volume of 95ml (Biochemica, Merck).

D-glucose ran to an Rf value of 0.47 and D-glucose-6-phosphate to an Rf value of 0.21. The plates were scraped directly into scintillation vials at the origin ± 5mm, at the mid point of the Rf values ± 5mm, and the top of the plate ± 5mm (as a background estimation). Five millilitres of scintillant was added to each scintillation vial, stored overnight, and counted in a Packard liquid scintillation counter.

3.3 DETERMINATION OF THE MICHAELIS CONSTANT AND MAXIMUM VELOCITY OF THE PLASMA MEMBRANE ASSOCIATED HEXOKINASE

Method

Plasma membranes in Tes/EDTA, were incubated at 3.9mg protein/ml in 80mM Tes buffer pH7.5, 5mM $MgCl_2$, 1mM ATP (or 1mM ADP), at D-glucose concentrations of 50µM, 500µM and 5mM, total volume 2ml. The assay was initiated by addition of D-glucose and incubated at 37°C with gentle agitation. Aliquots of 540µl were taken at 10 second, 20 second and 60 second intervals and pipetted directly into a plastic 1.5ml centrifuge tube containing 60µl of 80% (w/v) trichloroacetic acid. Denatured protein was removed by centrifugation at 20,000g, 5min, 20°C and 550µl of supernatant added to 750µl of diethyl ether in a 1.5ml plastic centrifuge tube. The two phases were vortex mixed, allowed to separate and the ether phase containing trichloroacetic acid discarded. The ether wash was repeated, and residual ether in the aqueous phase removed by standing the centrifuge tube in a fume cupboard for one hour.

Total D-glucose-6-phosphate was determined using the hexokinase marker enzyme assay, with the difference that enzyme activity was continued for 20 minutes until all D-glucose-6-phosphate had been utilised (determined by no increase in absorbance at 340nm). Total D-glucose-6-phosphate was calculated from the absorbance measurement.

4.0 PREPARATION OF PLASMA MEMBRANES FOR EXAMINATION BY ELECTRON MICROSCOPY

The trypanosome plasma membranes were fixed with both osmium tetroxide and glutaraldehyde. Osmium tetroxide was used primarily to bind lipid double bonds and hence crosslink neighbouring lipid molecules, but also to fix proteins via sulphydryl groups and side chain amino groups.

Glutaraldehyde was used for more efficient fixation of the plasma membrane proteins via side chain amino groups. Most importantly glutaraldehyde preserved microtubules (Weakley, 1981) important for plasma membrane identification.

Lead acetate was used to increase the contrast of the plasma membranes by binding the reduced osmium and also sulphydryl, tyrosyl , carboxyl (anion form)and ionised phosphate groups. Uranyl acetate was used to bind phosphate and carboxyl groups and was particularly useful in visualising the plasma membranes (Hayat, 1981). Cacodylate buffer replaced phosphate buffer to prevent precipitation of lead citrate stain.

Materials

Electron microscopy grade reagents (glutaraldehyde, osmium tetroxide (OsO_4), uranyl acetate, TAAB resin, lead citrate) and copper grids were supplied by the University of Bath Electron Microscopy Unit. Analar grade cacodylate (sodium dimethyl arsenic acid) was

supplied by Sigma Chemical Co., Poole, Dorset.

Method

When required plasma membrane sheets were collected at 7,500g, 30 seconds, throughout the procedure.

Plasma membrane pellet

Suspended in 4% v/v glutaraldehyde, 0.1M cacodylate buffer pH7.2, 12h, 20°C, then collected by centrifugation.

Supt. discarded — Pellet

Pellet washed thrice with 0.1M cacodylate buffer pH7.2, 20°C.

Supt. discarded — Pellet

Pellet resuspended in 1% (w/v) OsO_4, 1h, 20°C, centrifuged and washed thrice with DDH_2O, 20°C, to remove OsO_4, and cacodylate buffer, the latter to prevent precipitation of uranyl acetate.

Supt. discarded — Pellet

Pellet resuspended in 1% (w/v) aqueous uranyl acetate, 1h, 20°C, in the absence of light to prevent precipitation of uranyl acetate, washed twice with DDH_2O, then collected by centrifugation.

Supt. discarded

Pellet

Dehydrated, 20°C, in 30% (v/v) acetone twice (10min) 70% (v/v) acetone twice (10min) and 100% thrice, then collected by centrifugation.

Supt. discarded

Pellet

Pellet suspended in 50:50(v/v) acetone/TAAB resin, stood 20°C, 12h. Acetone/resin replaced by 100% resin, stood 48h, replaced by 100% resin 12h, and then hardened in an oven, 60°C, 48h.

Resin block

Thin sections taken from resin blocks with a glass knife and Reichart OM U3 cutting microtome, were placed on copper grids and stained with a saturated ethanolic solution of uranyl acetate, 5min (absence of light to prevent precipitation), followed by lead citrate (absence of CO_2 to prevent precipitation), 10min. Excess stain was washed off in DDH_2O.

Stained sections

Sections studied under Jeol 1WCX electron microscope.

5.0 SODIUM HYDROXIDE TREATMENT OF PLASMA MEMBRANES TO RELEASE THE MICROTUBULAR ARRAY

Materials

Sodium phosphate buffer (5mM) taken to pH8.0 with 1M sodium hydroxide. Sodium hydroxide solutions containing EDTA (2mM), dithiothreitol (0.2mM) added fresh on day of experiment), and sodium hydroxide concentrations of 5mM, 10mM and 15mM. Sodium phosphate buffer (10mM), adjusted to pH7.4 with 1M sodium hydroxide.

Method

This technique is based on that of Gorga and Lienhard (1981) developed for erythrocyte "ghosts".

T.b.brucei plasma membranes produced freshly on the day in PBS/EDTA buffer were washed thrice in 50ml of sodium phosphate buffer (5mM) pH8.0, 4°C, in an MSE 18 centrifuge, 7,500g, 10secs. Washed membranes were resuspended in sodium phosphate buffer (5mM) pH8.0, 4°C at a protein concentration of 4mg/ml.

Five volumes of sodium hydroxide solution were added to the trypanosome plasma membranes and allowed to stand at 4°C for 10 min. Membranes were collected at 48,000g for 30min, 4°C in a Beckman L5-65 ultracentrifuge, washed once with 50ml sodium phosphate buffer (10mM) pH7.4, and finally resuspended with 100µl of 10mM sodium phosphate buffer, 100mM D-glucose, 0.1mM EDTA, 0.1mM PMSF, pH7.4.

One hundred microlitres of suspension was incubated at room temperature for 30min, and then stored on ice until used. The remainder was taken to 3ml with 10mM sodium phosphate buffer, 100mM D-glucose pH7.4, and taken through the complete freeze/thaw vesiculation procedure and assayed for D-glucose transport as outlined in "preparation of plasma membrane vesicles", Section 5.2 and "plasma membrane and reconstituted proteoliposome infinite-trans sugar transport protocol", Section 10.3.

5.1 DEPOLYMERISATION OF THE PLASMA MEMBRANE PELLICULAR ARRAY OF MICROTUBULES BY CALCIUM ION TREATMENT

Materials

Sodium phosphate-glutamate buffer (20mM Na_2HPO_4, 100mM glutamic acid (BDH analar grade), 1mM mercaptoethanol (Sigma Chemical Co.), 0.1mM PMSF, 5µg/ml leupeptin), taken to pH7.0 with 1M sodium hydroxide. Calcium chloride was analar grade from BDH Chemicals Ltd. and defatted bovine serum albumin from Sigma Chemical Co. Before the experiment glassware to be used was stood overnight in concentrated nitric acid and then washed in double distilled water and dried before use.

Method

This technique primarily follows that of Dolan et al. (1986). Plasma membranes in PBS/EDTA or Tes/EDTA buffer were washed thrice in phosphate-glutamate buffer, collected at 4°C, 9,000g, 20s, in an Ole Dich refrigerated

centrifuge and finally resuspended in phosphate-glutamate buffer at a protein concentration of 2mg/ml, in a total volume of 2ml. The experiment was organised such that plasma membranes were subjected to calcium ion concentrations of 0, 0.1mM, 0.5mM and 1.0mM in the presence and absence of 1%(w/v) defatted bovine serum albumin. Membranes were incubated with and without calcium ions and defatted bovine serum albumin at 37°C for 30min with gentle agitation, and the depolymerisation process monitored by microscopic examination. The suspension was then cooled on ice for 20min and taken to 1mM EDTA by addition of 100mM EDTA. Membranes were collected by centrifugation at 9,000g, 10min, 4°C. Both supernatant (S1) and pellet (P1) were assayed for dihydrolipoamide dehydrogenase activity and total protein, as outlined previously. The supernatant (S1) from the process was subjected to further centrifugation at 20,000g, 1h, 4°C. The resulting pellet (P2) and supernatant (S2) were once again assayed for dihydrolipoamide dehydrogenase activity and total protein.

5.2 PREPARATION OF PLASMA MEMBRANE VESICLES

Materials

Sodium dihydrogen orthophosphate (BDH Chemicals Ltd.) was made up in double distilled water and taken to pH7.4 with 1M sodium hydroxide at concentrations of 5, 10, 30 and 100mM, containing 0.1mM PMSF, 0.1mM EDTA. Ethanol was analar grade from James Burroughs,

England, and solid carbon dioxide supplied by the Distillers Company Ltd., Coleshill, England.

Unlabelled and radiolabelled [6-^{3}H]-6-deoxy--D-glucose (8.13Ci/mmol) and [U-^{14}C]-1-deoxy-D-glucose (295 mCi/mmol) were synthesised by S. Game (1988). Plastic centrifuge tubes were purchased from Sarstedt Ltd.

Method

Plasma membranes produced as outlined previously were washed twice in 50ml of sodium phosphate buffer pH7.4, 4°C and collected at 7,500g , 10 sec, 4°C in an MSE 18 centrifuge. Membranes were finally resuspended in phosphate buffer pH7.4, 4°C, at protein concentrations of 5 to 10mg/ml, total volume 3 to 4ml.

Membranes were transferred to a glass Pyrex boiling tube and rapidly frozen in solid carbon dioxide/ethanol bath (-72°C). Membranes were thawed by standing the tube in a water bath at room temperature. The vesicle/supernatant fraction was separated from other fractions by transferral to a 4ml plastic centrifuge tube and centrifugation in an OLE DICH , CAMLAB refrigerated microcentrifuge (154), for 4min, 9,000g , 4°C.

In total five freeze/thaw cycles were found to be most efficient and the supernatants from each pooled and taken to 25ml with ice cold phosphate buffer. The vesicle fraction was collected at 100,000g, 4°C, 1h, in a Beckman Model L5-65 ultracentrifuge using a Beckman Type 60Ti rotor head.

The vesicle pellet was resuspended with 10 to 40µl of sodium phosphate buffer, pH7.4, 4°C, by rapidly "taking up" and expelling the fraction using a 200µl Gilson automatic pipette, until a homogeneous suspension resulted. This suspension was transferred to a sealed plastic Eppendorf 1.5ml microcentrifuge tube and stored at 4°C until required.

Sodium phosphate buffer concentrations varied between 5, 10, 30 and 100mM. For experiments where preloading of the vesicles with D-glucose was required the sodium phosphate buffer used for freeze/thawing and resuspending the final pellet contained 100mM D-glucose. In the case of 6-deoxy-D-glucose and 1-deoxy-D-glucose, preloading the vesicles was carried out by resuspending the pellet from high speed centrifugation with 300mM D-glucose analogue and adjusting the final concentration to 100mM with sodium phosphate buffer. Preloading took place by storing the vesicles at 4°C overnight, 12 to 16h, in a sealed Eppendorf 1.5ml microcentrifuge tube. D-glucose preloaded vesicles produced by standing with D-glucose overnight or by inclusion of D-glucose in the sodium phosphate buffer during freeze thawing demonstrated identical D-glucose infinite trans-transport with time.

6.0 PREPARATION OF ERYTHROCYTES

Erythrocytes were prepared according to the method of Levine et al. (1971). Outdated human transfusion blood (1-3 weeks old) was separated from plasma and acid-citrate-dextrose by washing with five volumes of phosphate buffer (containing 12.5mM Na_2HPO_4 and 154mM NaCl, taken to pH7.2 with 1M sodium hydroxide) followed by centrifugation at 2,500g, 10min, 20°C. The supernatant, white cells and platelets were removed by aspiration and the erythrocytes washed a further four times in phosphate buffer at 20°C. The erythrocytes were then left in a packed condition.

6.1 PREPARATION OF ERYTHROCYTE "GHOSTS"

Packed erythrocytes prepared as above at 20°C were lysed by plunging them into 15-25 times their volume of ice cold haemolysing buffer (containing 5mM Na_2HPO_4, 1mM EDTA, and 0.1mM PMSF, taken to pH8.0 with 1M sodium hydroxide) according to the method of Dodge et al. (1963). The suspension was stirred gently but thoroughly and left at 4°C for 20min. The membrane suspension was collected by centrifugation at 16,000g (6 x 300ml angle rotor, Sorvall RC-5B centrifuge) at 4°C for 20min with the centrifuge brake off. The supernatant was aspirated off and discarded, the fluffy membrane pellet removed with a pasteur pipette, and the hard pellet removed and discarded.

The fluffy membranes were washed once in ice

cold haemolysing buffer, collected by centrifugation as previously, the supernatant discarded and the fluffy membrane pellet resuspended in ice cold phosphate buffer. Two further washes of the membranes were carried out at 4°C in phosphate buffer, and the final fluffy pellet stored at -90°C until required.

7.0 SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

Materials

Acrylamide, N,N'-methylene bis acrylamide (Electran grade, purified for electrophoresis) and sodium dodecyl sulphate (SDS, specially pure) were from BDH Chemicals. Boric acid and Trizma base (analar grade) and glycerol (SLR) were from Fisons PLC. Ammonium persulphate (98% crystalline), N,N,N,N-tetramethylethylene diamine (TEMED), Brilliant Blue R (C142660), bromophenol blue (3',3",5',5"-tetrabromophenol sulfonphthalein), mercaptoethanol and molecular weight standards (myosin (205,000), β-galactosidase (116,000), phosphorylase b (97,000), bovine serum albumin (66,000), egg albumin (45,000) and carbonic anhydrase (29,000)) were all from Sigma Chemical Co. Ltd. The gel apparatus and water proof tape were from Pharmacia.

30% acrylamide solution

Thirty grams of acrylamide and 0.8g of N,N'-methylene bis acrylamide were dissolved in 100ml of double distilled water to give 30%(w/v) acrylamide and 0.8%(w/v) N,N'-methylene bis acrylamide.

Gel buffer

Trizma base (90.86g, 1.5M) and SDS (2.0g, 0.4% (w/v)) were dissolved in 500ml of double distilled water and taken to pH8.8 with concentrated hydrochloric acid.

20% (w/v) acrylamide solution	5% (w/v) acrylamide solution
5ml gel buffer	5ml gel buffer
13.33ml of 30% acrylamide	3.33ml 30% acrylamide
1.67ml double distilled water	11.67ml double distilled water
Total volume for each solution	20ml

Electrode buffer

Electrode buffer consisted of Tris (25mM), boric acid (40mM) and SDS (0.1%(w/v)) in a total volume of 5 litres, at pH8.3.

Gel stain and destain

Gel stain consisted of 0.2% (w/v) Coomassie Brilliant Blue R in acetic acid:ethanol:double distilled water (1:3:6) and destain the same as stain without the Coomassie Brilliant Blue R.

Method

Glass gel plates (20cm x 18cm x 0.2cm) were soaked in 2M potassium hydroxide in 95% ethanol overnight, rinsed with double distilled water and soaked overnight in 1% (v/v) decon solution. They were then rinsed thoroughly in double distilled water and dried in a warm oven.

Two plates, separated by 1mm teflon spacers were taped together with Pharmacia waterproof tape, and clamped vertically. A fast setting 20% acrylamide solution (2ml) containing 5µl of TEMED and 15µl of 10% (w/v) ammonium persulphate was used to seal the gel base. Eighteen mls of 20% acrylamide was added to one

chamber of a linear gradient mixer, and 20ml of 5% acrylamide to the other, and to both 30µl of TEMED and 90µl of 10% ammonium persulphate added with mixing. The acrylamide was pumped into the gel space by a peristaltic pump, (such that the 20% acrylamide was at the bottom of the gel and 5% acrylamide at the top), via hard rubber tubing, so that the tubing could be maintained just above the gel slab surface. This pouring process normally took approximately 15min.

Wells were produced in the 5% acrylamide using an eight tooth teflon comb placed in the gel before setting occurred. Double distilled water was layered onto the gel to avoid evaporation, and any shrinkage of gel during setting remedied by addition of 5% acrylamide solution.

Samples to be loaded on the gel were precipitated with trichloroacetic acid (5%(w/v)) and protein collected at 9,000g, 20°C for 5sec in a microfuge (Dolan et al., 1986). The protein pellet was resuspended with 100µl of gel buffer, containing 1.0% (w/v) SDS and 1.0% (v/v) mercaptoethanol, to give total protein of up to 200µg, and boiled for 3 min. One grain of bromophenol blue tracker dye was added to each protein sample and the samples applied to the gel.

The gels were run in a Pharmacia electrophoresis tank containing electrode buffer, until the tracker dye reached the base of the gel. This usually took 14 to 18h at a current of 22 to 28mA. Each gel was stained for 6h in stain and then destained until protein bands

were clearly visible against a clear background, normally 36 to 48h.

8.0 EXTRACTION OF TOTAL TRYPANOSOME LIPID FROM TRYPANOSOMES

Materials

Analar grade methanol and chloroform were supplied by James Burroughs and May & Baker, England, respectively. Chloroform was tested for phosgene according to the method of Bergelson (1980) by the addition of 1ml of solvent to 1ml of 1% (w/v) p-dimethyl-amino-benzaldehyde (from Sigma Chemical Co.) in double distilled water. Any phosgene present would produce an intense yellow colour. This was not found in any of the chloroform tested.

Method

Lipid extraction followed the method of Bligh and Dyer (1959) assuming the trypanosome pellet was 80% water. Whole trypanosomes in Krebs Ringer phosphate buffer, 10mM D-glucose pH8.0 were used fresh or after thawing from storage at -90°C in Krebs Ringer phosphate buffer, 10mM D-glucose, 12% (w/v) glycerol. Trypanosomes were put in a 50ml MSE plastic centrifuge tube, washed twice in 50ml of Krebs Ringer phosphate buffer pH8.0, 4°C , collected by centrifugation in an MSE 18 centrifuge at 10,000g, 5min, 4°C, and the supernatants discarded.

Double distilled water was added to the packed trypanosome pellet according to the pellet weight, see table on following page.

Weight of tissue (g, wet wt.)	Vol of H_2O for homogenisation	Vol. MeOH/$CHCl_3$ for shaking	Final step vol. of NaCl & $CHCl_3$
1	0.5	5	1.7
2	1.0	10	3.3
3	1.4	14	4.7
4	1.9	19	6.3
5	2.4	24	8.0

The pellet and double distilled water were vortex mixed at room temperature for 20sec. Lipid was solubilised by addition of methanol/chloroform (2:1 by volume) to the trypanosome/double distilled water mixture, the volume determined by reference to the table above, and the mixture vortexed for 20 seconds at room temperature.

Insoluble material was removed by filtration through Whatman No 1 filter paper (prewashed with methanol/chloroform 2:1 by volume) on a Buchner funnel (prewashed with 2:1 by volume methanol/chloroform) under suction, and the resulting filtrate transferred to a MSE 50ml plastic centrifuge tube. Double distilled water containing 0.58% (w/v) sodium chloride (Voorheis et al., 1979), and chloroform were added to the lipid extract in volumes as outlined in the table above. After vortex mixing for 5 seconds the resulting milky emulsion was separated into two distinct phases by centrifugation in an IEC Centra 3R bench centrifuge at 3,000rpm, 5min, room temperature. The chloroform phase was removed and kept to one side, whilst the methanol/sodium chloride solution

9.0 RECONSTITUTION OF TRYPANOSOME AND HUMAN ERYTHROCYTE PLASMA MEMBRANE PROTEINS

Materials

Soybean L-α-phosphatidyl choline (L-α-lecithin) TypeII S egg yolk L-α-phosphatidyl choline (L-α-lecithin) Type V-EA, 99% pure, n-octyl-β-D-glucopyranoside, egg yolk L-α-lysolecithin, catalase (bovine liver, 11,000U/mg protein, glucose oxidase (Aspergillus niger, type II, 38U/mg protein), decanoyl-N-methyl-glucamid (N-methyl glucamid, MEGA 10) and sodium cholate were supplied by Sigma Chemical Co. Non-polar polystyrene beads, biobeads SM2 were purchased from Biorad Laboratories Ltd., Watford, England, and a Decon FS 100 frequency sweep, 100 watt, 1.5 litre bath sonicator from Decon Laboratories Ltd., Hove, England.

Zwittergent 14 was supplied by Calbiochem-Behring Corp. La Jolla, California, dialysis tubing (size 1-8/32") by Medicell International Ltd., London, England, and Sepharose Cl-6-B by Pharmacia Fine Chemicals A.B.. Uppsala, Sweden.

9.1 FREEZE/THAW AND SONICATION RECONSTITUTION OF PLASMA MEMBRANE PROTEINS

9.1.1 Detergent extracted proteins

Method

The technique principally follows that developed by Kasahara and Hinkle (1976). T.b.brucei plasma membranes

and human erythrocyte ghosts, produced as outlined previously, were washed thrice in Tris/HCl buffer (10mM) pH7.4, or sodium phosphate buffer (10mM) pH7.4, at 4°C as outlined previously, and finally resuspended in the buffers in the presence and absence of EDTA (1mM), 2-mercaptoethanol (1mM), PMSF (0.1mM) and leupeptin (5µg/ml).

Membranes (2mg of protein) were incubated with 0.5% (w/v) Triton X-100 in 100mM buffer pH7.4, at 4°C to give a final protein concentration of 2mg/ml. After 20 min incubation at 4°C, with occasional inversion to mix, the insoluble material was removed by centrifugation in a refrigerated OLE DICH CAMLAB microcentrifuge, 20,000g, 1h, 4°C, and discarded. The clear supernatant was added to Biobeads SM2 pre-equilibrated in 10mM buffer pH7.4 (0.3g beads/ml supernatant) and agitated gently at 4°C overnight to remove the detergent.

Biobead treated extract, 0.5ml, was added to 250µl of preformed liposomes (Soybean L-α-lecithin 50mg/ml vortex mixed and sonicated to clarify in 10mM buffer pH7.4) to give 15 to 20mg of lipid and 300 to 600µg of protein in 750µl. This mixture was frozen rapidly in an ethanol/dry ice bath, and thawed at room temperature. Resulting proteoliposomes were sonicated in a Decon FS 100 bath sonicator (adjusted for maximum agitation of the proteoliposomes), for 10 seconds, and then stored on ice until required.

The above procedure of solubilisation and reconstitution was applied to plasma membranes extracted

with Zwittergent 14 at 2 and 3mM final concentrations and to n-octyl-β-D-glucopyranoside at 50mM final concentration in place of 0.5% (w/v) Triton X-100. Zwittergent 14 was removed by Biobeads SM2 as for Triton X-100, and n-octyl-β-D-glucopyranoside by dialysis for 12h at 4°C, against 1 litre of 10mM Tris/HCl buffer, pH7.4.

The procedure was modified to assay a range of concentrations of Zwittergent 14, Triton X-100 and L-α-lysolecithin (membranes treated for 10min at room temperature followed by incubation of the extract with Biobeads SM2 as for Zwittergent 14 and Triton X-100) including 100mM D-glucose in all the buffers. This produced proteoliposomes preloaded with 100mM D-glucose. The proteoliposomes were diluted with 25ml of 10mM sodium phosphate buffer, 0.1mM PMSF, 0.1mM EDTA, 100mM D-glucose pH7.4, 4°C, in a Beckman 25ml ultracentrifuge tube, and collected by centrifugation at 100,000g, 4°C in a Beckman L65 ultracentrifuge using a Beckman Ti 60 rotor.

The proteoliposome pellet was resuspended with 20µl of 10mM sodium phosphate buffer pH7.4, 0.1mM PMSF, 0.1mM EDTA, 100mM D-glucose, 4°C, to give a homogeneous suspension of proteoliposomes. Proteoliposomes were kept on ice until required.

9.1.2 Non-detergent extracted proteins

T.b. brucei plasma membrane vesicles produced by the freeze/thaw procedure outlined previously, in

10mM sodium phosphate buffer pH7.4, 0.1mM EDTA, 0.1mM PMSF, 100mM D-glucose, were added to preformed Soybean L-α-lecithin vesicles, sonicated to clarity in 10mM sodium phosphate buffer pH7.4, 0.1mM EDTA, 0.1mM PMSF, 100mM D-glucose to give a final protein concentration of 1mg/ml and lipid concentration of 20mg/ml in a total volume of 1ml.

The mixture was frozen in ethanol/dry ice bath and thawed at room temperature. Proteoliposomes formed by the procedure were sonicated for 10 seconds, collected by ultracentrifugation as outlined previously, and resuspended with 20µl of 10mM sodium phosphate buffer pH7.4, 0.1mM EDTA, 0.1mM PMSF and 100mM D-glucose.

9.2 DETERGENT DIALYSIS RECONSTITUTION PROCEDURE

Method

This technique principally follows that of Shelton and Langdon (1983). Membranes (erythrocyte and T.b.brucei) were washed thrice in 10mM sodium phosphate buffer, 0.1mM 0.1mM EDTA, 0.1mM PMSF , pH7.0, as outlined previously, at 4°C. To 0.5ml of membrane (2.5 to 3.5 mg of protein/ml) was added 0.5ml of 10mM sodium phosphate buffer pH7.0, 0.1mM EDTA, 0.1mM PMSF, 340mM N-octyl-β-D-glucopyranoside. The mixture was stood on ice for 20min with occasional inversion to mix, followed by centrifugation, 20,000g, 4°C, 20min, in an OLE DICH refrigerated centrifuge, to remove insoluble material. The clear supernatant (approximately 1ml) was added to 1ml of 10mM sodium

lipid. Soybean L-α-lecithin was sonicated to clarity, T.b.brucei whole lipid prepared as outlined previously, and the egg yolk L-α-lecithin were both vortex mixed for 10min and sonicated for 2h under nitrogen. The lipid solutions contained the lipid at 50mg/ml in double distilled water, 2mM mercaptoethanol.

The lipid and detergent extract was inverted to mix and stood on ice for 15min before being rapidly injected through a Sabre 0.6 x 30mm hypodermic needle on a 2ml plastic syringe, into 20ml of 100mM potassium phosphate buffer pH7.0 containing 100mM D-glucose at 20°C. Resulting proteoliposomes were incubated at 20°C for 20min and collected by centrifugation at 100,000g, 1h, 4°C in a Beckman L65 ultracentrifuge, and Ti 60 rotor.

Proteoliposomes were resuspended with 20µl of 100mM potassium phosphate buffer, pH7.0, containing 100mM D-glucose and stored on ice until assayed.

9.3.2 Reconstitution from decanoyl-N-methyl-glucamid and sodium cholate extracts of trypanosome plasma membranes

The reconstitution principle follows that outlined for n-octyl-β- D-glucopyranoside with several modifications. Trypanosome plasma membranes resuspended in 10mM sodium phosphate buffer pH7.4, 0.1mM EDTA, 0.1mM PMSF, 100mM D-glucose, were incubated with decanoyl-N-methyl-glucamid (MEGA 10) at concentrations of 0, 0.25, 0.5 and 1.0%

(w|v) detergent (protein concentration 2mg|ml, total volume 1ml) at 20°C for 20min, in 10mM sodium phosphate buffer, pH7.4, 0.1mM EDTA, 0.1mM PMSF, 100mM D-glucose. Incubation with sodium cholate was carried out at 0, 0.5, 1.0, 1.5 and 2.0% (w/v) sodium cholate at 4°C, with other conditions identical to decanoyl-N-methyl--glucamid.

Insoluble material was removed by centrifugation, 20,000g, 1h, 4°C (for sodium cholate) and 20°C for decanoyl-N-methyl-glucamid. One millilitre of supernatant was added to 250µl of Soybean L-α-lecithin (20mg of lipid) sonicated to clarity in a Decon FS 100 bath type sonicator in 10mM sodium phosphate buffer, pH7.4, 0.1mM EDTA, 0.1mM PMSF, 100mM D-glucose and allowed to stand at 4°C, 1h, for sodium cholate extractions, and 20min, 20°C for decanoyl-N-methyl-glucamid (MEGA 10) extractions. After standing for the allotted times, the proteoliposomes were rapidly diluted by injecting the protein:lipid: detergent mixture into 20ml of 10mM sodium phosphate buffer pH7.4, 0.1mM PMSF, 0.1mM EDTA, 100mM D-glucose, at room temperature (for both detergents). Proteoliposomes were collected by centrifugation, 100,000g, 1h, 4°C, in a Beckman L5-65 ultracentrifuge and Beckman Ti60 rotor, and resuspended in 20µl of 10mM sodium phosphate buffer pH7.4, 0.1mM EDTA, 0.1mM PMSF, 100mM D-glucose, and stored on ice until assayed.

9.4 DIRECT INCORPORATION RECONSTITUTION PROCEDURE FOR TRYPANOSOME PLASMA MEMBRANE PROTEINS

The direct incorporation procedure is based on that developed by Eytan et al. (1975) utilising L-α-lysolecithin from egg yolk.

Plasma membrane vesicles produced by the freeze/thaw technique outlined previously, in 10mM sodium phosphate buffer pH7.4, 0.1mM EDTA, 0.1mM PMSF, 100mM D-glucose, were added to a lipid mixture containing soybean L-α-lysolecithin (10% (w/w) of the total phospholipids) in 10mM sodium phosphate buffer pH7.4, 0.1mM EDTA, 0.1mM PMSF, 100mM D-glucose, to give a final concentration of 20mg/ml and protein concentration of 1mg/ml in 1ml.

After incubation for 12h at 4°C with gentle agitation, the proteoliposomes were diluted with 20ml of ice cold 10mM sodium phosphate buffer pH7.4, 0.1mM EDTA, 0.1mM PMSF, 100mM D-glucose, and collected by centrifugation at 100,000g, 4°C, 1h, in a Beckman L65 ultracentrifuge.

The proteoliposome pellet was resuspended once again with 20µl of the 10mM sodium phosphate buffer pH7.4, and collected as previously indicated. The resulting proteoliposomes were resuspended with 20µl of 10mM sodium phosphate buffer pH7.4, 0.1mM EDTA, 0.1mM PMSF, 100mM D-glucose, and stored on ice until assayed.

with 1ml of KRB pH8.0 (no D-glucose) and collected at 16,000g av, 20sec, 4°C. Cells were resuspended by vortex with 30µl of KRB, and 20µl of these added to 5µl of 6-^{3}H-6-deoxy-D-glucose in another 4ml plastic tube at room temperature (20°C). 6-deoxy-D-glucose concentration was 129µM with a total radioactivity of 2.4µCi per tube.

The cells were allowed to accumulate 6-deoxy-D-glucose for 1min at room temperature before being diluted 1 in 20 with 475µl of KRB, or KRB containing the inhibitor to be used. Time points were taken at various times between 3 and 60 secs by aliquotting 100µl of cell suspension into 2ml of ice cold KRB pH8.0, 0.1% (w/v) phloridzin (2.2mM) in a 4ml plastic centrifuge tube. Cells were collected at 16,000g, 4°C, 20sec, the supernatant discarded and cell pellet resuspended by vortex with 1ml of ice cold KRB pH8.0, 0.1% (w/v) phloridzin. Cells were once again collected at 16,000g,4°C, 20sec, and the supernatant discarded.

Zero time points were determined by dispensing 5µl of the cells immediately after the 60sec. pre-incubation straight into 2ml of ice cold KRB pH8.0, 0.1% (w/v) phloridzin and then washing as outlined above. Background contamination was determined by dispensing 5µl of cells treated as above, except 5µl of KRB replaced the 5µl of 6-deoxy-D-glucose, straight into 2ml of ice cold KRB pH8.0, 0.1% (w/v) phloridzin containing the 5µl of tritium labelled 6-deoxy-D-glucose. The cells were then washed as previously outlined.

After washing, 500µl of double distilled water

was added to the cell pellet, and 450µl of the resuspended, lysed cells added to 5ml of PPO/toluene/Triton X-100 scintillant in a scintillation vial.

Samples were stored overnight and counted in a LKB liquid scintillation counter.

10.2 ZERO-TRANS ENTRY SUGAR TRANSPORT INTO TRYPANOSOMES PROTOCOL

Isolated trypanosomes in KRB pH8.0, 10mM D-glucose were dispensed into 4ml polypropylene centrifuge tubes to give 5×10^7 cells per tube. The cells were washed free of D-glucose by the addition of 1ml of ice cold KRB pH8.0, followed by centrifugation at 16,000g, 4°C, 20sec, in an OLE DICH refrigerated microcentrifuge. The cell pellet was resuspended in 1ml of ice cold KRB by gentle vortex, and the cells collected as above. Eighty microlitres of KRB (20°C) were added to the cell pellet, the tube gently vortexed, and the cell suspension added to 20µl of 6-deoxy-D-glucose solution. The 20µl consisted of 6-^{3}H-6-deoxy-D-glucose (1.2µCi) and 5µl of 20 times concentrated unlabelled 6-deoxy-D-glucose taken to 20µl with KRB pH8.0, to give a final 6-deoxy-D-glucose concentration of 129µM in the tube after addition of the cells.

Time points were measured from the moment of addition of cells to the 6-deoxy-D-glucose, and terminated by pipetting the total assay solution into 2ml of ice cold KRB pH8.0 containing 0.1% (w/v) phloridzin (2.2mM). Cells were collected by centrifugation at 16,000g, 20sec,

4°C, and the supernatant aspirated off carefully to avoid leaving behind any droplets on the tube sides. One ml of KRB pH8.0, 0.1%(w/v) phloridzin (4°C) was added, and the cell pellet resuspended by gentle vortex. Trypanosomes were collected by centrifugation as above, the supernatant aspirated off, and the cell pellet resuspended in 500µl of double distilled water. Four hundred and fifty microlitres of lysed cells were added to 5ml of scintillant and radioactivity determined on a Packard scintillation counter.

Extracellular radiolabel was determined by direct addition of the cells resuspended in 80µl of KRB pH8.0, to 2ml of KRB, 0.1% (w/v) phloridzin containing 20µl of 6-deoxy-D-glucose solution. This value for "zero" transport was subtracted from each time point in the assays. For time points less than 10 seconds a metronome counting two beats per second was used.

In assays where inhibitors were used, the inhibitor was included in the 20µl of 6-deoxy-D-glucose solution such that the required concentration was achieved upon addition of 80µl of cells.

10.3 PLASMA MEMBRANE VESICLES AND RECONSTITUTED PROTEOLIPOSOMES INFINITE-TRANS SUGAR TRANSPORT PROTOCOL

Trypanosome plasma membrane vesicles and reconstituted proteoliposomes produced as outlined previously, preloaded with 100mM sugar (D-glucose,

under tap pressure suction, by application to the centre of a 0.22μm Millipore GSWP filter (presoaked in stopping solution) on a Millipore filter turret. Filters and vesicles were washed free of extravesicular radioactive sugar by the progressive application of 5ml of ice cold stopping solution, such that the portion of the filter to which the vesicles had been applied remained moist, until all of the 5ml had been applied.

After washing, the filters were taken from the turret, placed in a scintillation vial containing 5ml of scintillant, and stood overnight for complete extraction of the filter. Radioactivity was counted in a Packard liquid scintillation counter.

Zero time points and background radioactivity were determined by aliquotting 2μl of vesicles directly into 1ml of ice cold stopping solution. Two hundred microlitres of diluting buffer containing radiolabelled sugar was added to the stopped vesicles, and 1ml immediately filtered, washed, and radioactivity counted as outlined previously. These background values were subtracted from the assay values. For greater accuracy small volumes of vesicles were dispensed using a Ultipette 1-10μl pipette.

To try to improve vesicle retention on the filters, a comparison was made between filters soaked in stopping buffer containing 1mg/ml poly-L-lysine and filters soaked in stopping buffer only.

10.4 PROTEOLIPOSOME ZERO-TRANS SUGAR TRANSPORT PROTOCOLS

10.4.1 Proteoliposomes produced by the freeze/thaw/sonication method

D-glucose transport assays included ^{14}C radiolabelled D-glucose, 0.3µCi for erythrocyte derived proteoliposomes, and 1.0µCi for trypanosomes plasma membrane derived proteoliposomes, 1.0 to 3.0mg of lipid and 30 to 87µg protein per assay.

Assays were started by the addition of 108µl of vesicles at 20°C to 12µl of D-glucose solution in 10mM buffer (Tris/HCl pH7.5 or sodium phosphate pH7.4). The assay was terminated after the desired time by rapidly pipetting 100µl of assay into 1ml of ice cold 10mM buffer (containing 0.5mM mercuric chloride for erythrocyte proteoliposomes).

Filtering, washing and liquid scintillation counting were carried out as outlined in the infinite-trans sugar transport protocol.

10.4.2 Proteoliposomes produced by the detergent dialysis method incorporating glucose oxidase and catalase

Five hundred microlitres of proteoliposomes in 10mM sodium phosphate buffer pH7.0, 0.1mM PMSF, 0.1mM EDTA were dispensed into a Clark type oxygen electrode and allowed to reach 20°C. The volume was made up to 1ml with 10mM sodium phosphate buffer pH7.0, 0.1mM PMSF, 0.1mM EDTA, and ten times concentrated

D-glucose solution, to give the required D-glucose concentration, at 20°C. The mixture was stirred gently during the assay and D-glucose transport into the proteoliposomes monitored by the reduction in oxygen according to the following scheme.

$$2\ \text{D-glucose} + 2O_2 \xrightarrow[\text{oxidase}]{\text{glucose}} 2\ \text{D-gluconic acid} + 2H_2O_2$$

$$2\ H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

Oxygen consumption was traced on a Rikadenki chart recorder, from which the initial rates at a range of D-glucose concentrations were calculated.

Inhibition of D-glucose transport by p-chlorophenyl-mercaptosulphonic acid (p-CPMSA) was carried out by adding p-CPMSA in 10mM sodium phosphate buffer pH7.0, 0.1mM PMSF, 0.1mM EDTA, to the proteoliposomes in the oxygen electrode, to give a final concentration of 1mM. After preincubation for 1min the assay was started by addition of 10 times concentrated D-glucose solution, to give the final desired D-glucose concentration.

RESULTS

1.0 PRODUCTION OF A TIGHTFITTING DOUNCE TYPE HOMOGENISER

Three designs of teflon homogeniser tip, a sphere, a rod (with rounded end), and a cone, were tested to produce optimum breakage of the swollen trypanosomes. Production of the sphere and rounded rod required that teflon be shaved from the tip such that the diameter of the rod or sphere decreased with each cut, fig. 16 A and B. The lathe available was not sensitive enough to produce the optimum fit. Both the sphere and rod generated large amounts of friction when travelling down the homogeniser (making homogenisation difficult), without providing a small enough clearance between the tip and the glass pestle to satisfactorily break the swollen trypanosomes.

To overcome this problem the tip shape was altered to a cone. Teflon was removed from the surface opposite to the tip, *ie.* not directly off the diameter of the cone, fig. 16 C. With this procedure, removing a thin layer of teflon from the face opposite the tip, within the limits of the lathe available, removed a much smaller amount off the diameter of the cone. In this fashion a homogeniser tip capable of effectively breaking up to 95% of the swollen trypanosomes (as determined by light microscopy) with one stroke of the homogeniser was designed.

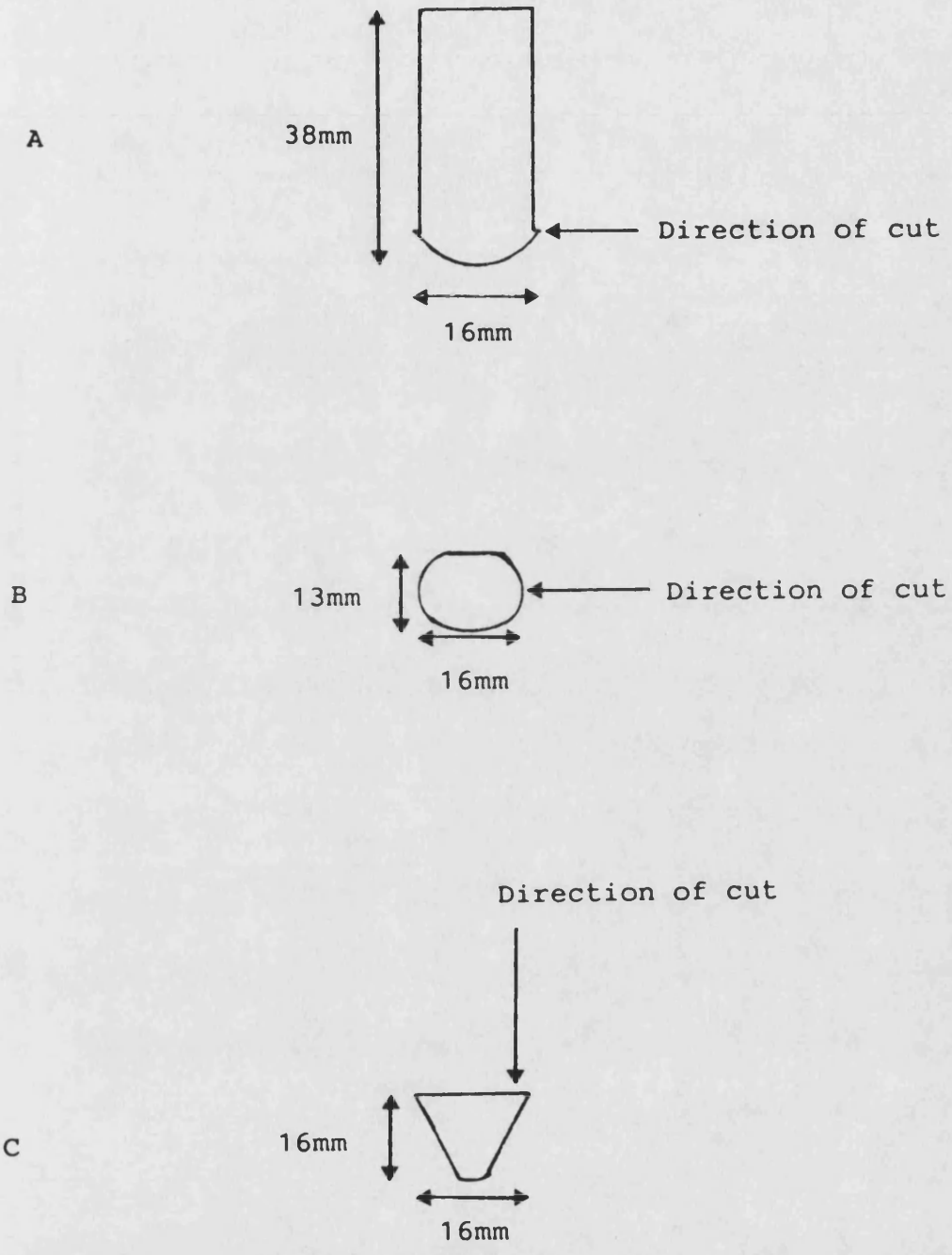


Fig. 16 Showing dimensions and direction of cut by the lathe to produce the optimum homogeniser tip for the round ended rod (A), the sphere (B) and the cone (C).

2.0 PLASMA MEMBRANE ISOLATION

The method of Voorheis (1979) was used, but modified slightly in several aspects, for isolation and purification of the plasma membranes In the Tes buffer system the swelling of the trypanosomes was carried out with swelling buffer containing 1mM Tes, to maintain buffering capacity in the cell suspension after dilution, and 1mM EDTA and 2-mercaptoethanol to maintain their respective concentrations. Leupeptin was included in the swelling buffer to inhibit thiol proteases present (Voorheis, personal communication). An extra wash of pellet P1 was included to remove free enzymes present because of the high number of cells used (2 to 4 x 10^{10}) per experiment, and to provide a second opportunity for leupeptin to neutralise thiol proteases.

The Tes buffer system was replaced by phosphate buffered saline to provide a continuity of buffer systems based on phosphate, from the isolation of the trypanosomes to the reconstitution of the membrane proteins. The concentration of EDTA was decreased to 0.1mM to avoid any disruption of membrane structure by chelation of divalent metal ions (particularly calcium), associated with maintaining membrane integrity. Mercaptoethanol was not used in case it protected the thiol proteases present.

Apart from the above, the isolation method followed that of Voorheis (1979). Fig. 17 shows a photograph of the 40-60% (w/v) sucrose gradient (the gradient

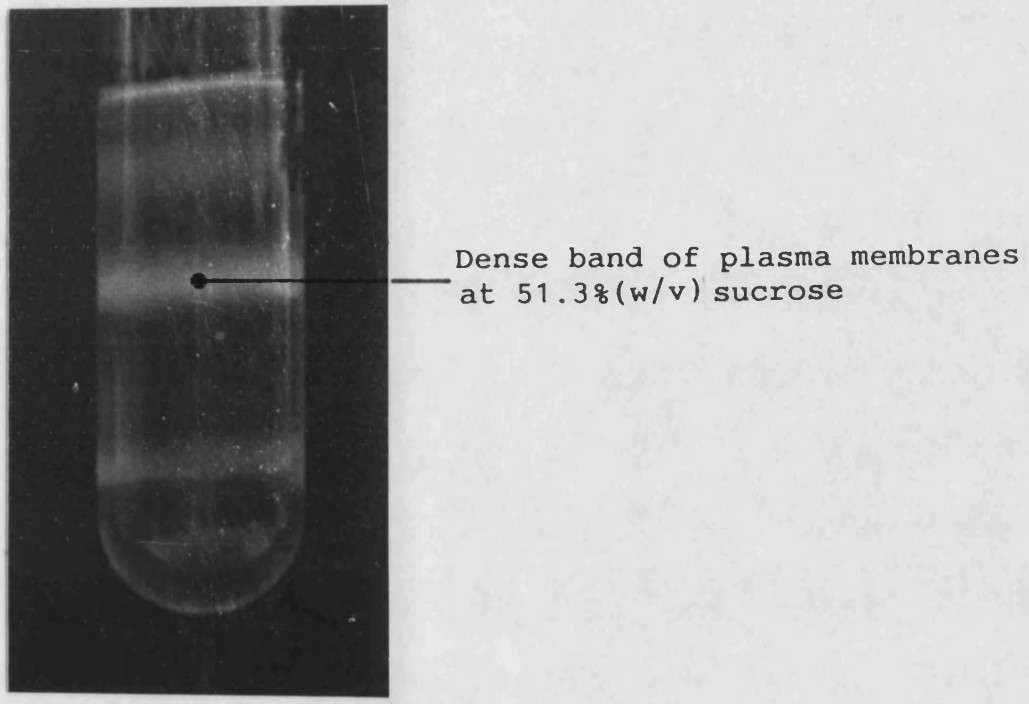


Fig. 17 40-60% (w/v) sucrose gradient showing the band at 51.3% (w/v) sucrose corresponding to the plasma membranes.

included a 60% (w/v) sucrose cushion (5ml) but no 52% (w/v) sucrose layer). The dense band of plasma membranes is shown at 51.3% (w/v) sucrose.

Total protein estimations of the cell homogenate, pellet P1, supernatant S1, pellet P3 and pellet P4 (the final membrane pellet) from four experiments are given in milligrams of protein for every 1 x 10^{10} cells used in the purification, in the table below.

Sample	Homog.	P1	S1	P3	P4
Tot. prot. mg/1x10^{10} cells	83±3.9	53.5±3.4	36.1±3.8	40.9±4.9	10.8±2.0
% total homog. protein	100	64.5	36.3	49.3	13.0

The protein banding patterns of whole cells and plasma membranes are shown in fig. 18.

2.1 MARKER ENZYME ACTIVITIES OF PLASMA MEMBRANE PURIFICATION FRACTIONS

The enzyme specific activities are expressed as µmol of substrate utilised/mg protein/min except for myokinase which represents in the case of ADP production, the formation of 2ADP, from ATP and A5MP, and in the case of ATP production, the formation of 1ATP from 2ADP.

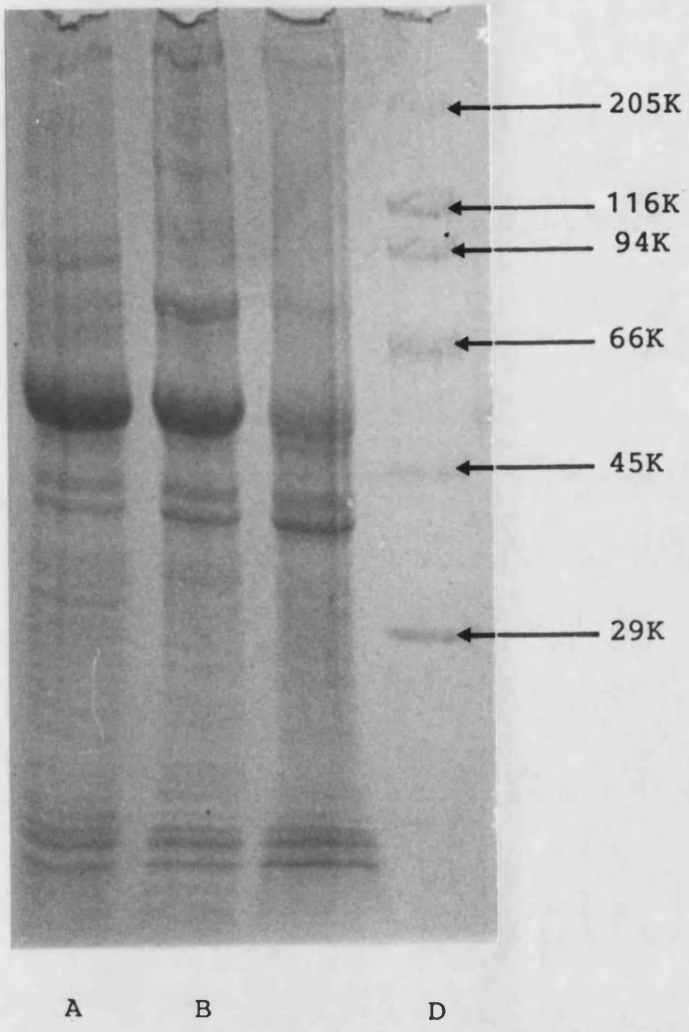


Fig. 18. 10-20% (w/v) SDS-PAGE gel of whole cells, lane (A) and purified plasma membrane, lane (B). Lane D is the molecular weight markers, with molecular weights as indicated.

Total enzyme activities are expressed in μmoles substrate/ min with the reservations outlined above for myokinase activity. Table 6 summarises the specific activities, total activities and % of the total enzyme activity, taking the homogenate value ± Triton X-100 0.1% (w/v) (whichever is larger) as the 100% value. For greater ease of reference the % total activity and specific activities have been drawn as bar charts in fig. 19.

Over 50% of the total pyruvate kinase and malate dehydrogenase activities in the homogenate were released into the S1 fraction, with less than 15% being retained in the pellet P1. The specific activities of the enzymes also decreased to 25% or less of the homogenate value, whilst increasing in the supernatant fraction by a factor of 1.3. For pyruvate kinase,treatment of the pellet P1 with DNAase reduced residual total activity to between 48 and 60% of the P1 value. This was reduced to 2.5% of the total homogenate value in pellet P4 with a specific activity 17% of the homogenate value. Some latency of pyruvate kinase existed in the pellet fractions producing no more than a 1.8-fold increase in activity when treated with Triton X-100. By contrast the addition of Triton X-100 to the homogenate and supernatant S1 decreased the total activity (to zero in S1) and may well have inactivated the enzyme. It has been suggested by Flynn and Bowman (1980) that pyruvate kinase is unstable in aqueous solution. Malate dehydrogenase mirrored pyruvate kinase in that total activity in the final pellet P4 was only 2.0% of the homogenate value and the specific

Table 6 Specific activity, total activity and % total activity of marker enzymes in fractions taken from the plasma membrane purification procedure ± 0.1% (w/v) Triton X-100. (Spec. Act.=μmoles substrate/mg protein/min & tot. act.=μmoles substrate/min). The number of determinations are in brackets. All replicate values have standard deviations not exceeding 28% of the given value. A - represents no determination.

ENZYME / ± TritonX-100 / SAMPLE		PYRUVATE KINASE		MALATE DEHYDROGENASE		D-G-6-Pase	
		-	+	-	+	-	+
H	Spec act.	0.058 (3)	0.044(3)	0.36(3)	-	0.0077(1)	0.0088(1)
	Tot act	11.7	9.0	40.6	-	1.9	2.1
	% Tot act	100	76.9	100	-	90.4	100
P1	Spec act.	0.0061(2)	0.011(2)	0.09(3)	-	0.0026(1)	0.0069(1)
	Tot act	0.9	1.7	5.75	-	0.37	1.0
	% tot act	7.7	14.5	14.2	-	17.6	47.6
S1	Spec act	0.075(2)	0(3)	0.55(3)	-	0.0098(1)	0.0155(1)
	Tot act	6.2	0	26.1	-	0.8	1.2
	% tot act	53	0	64.3	-	38.1	57.1
P3	Spec act	0.0038(2)	0.0058(2)	-	-	-	-
	Tot act	0.54	0.82	-	-	-	-
	%tot act	4.6	7.0	-	-	-	-
P4	Spec act	0.0055(3)	0.0099(3)	0.02(3)	-	0.002(1)	0.0048(1)
	Tot act	0.16±	0.29	0.81	-	0.09	0.22
	% tot act	1.4	2.5	2.0	-	4.3	10.5

Table 6 (continued) Values marked ° for myokinase represent the values for the production of ATP from 2ADP.

ENZYME ±Triton X-100 / SAMPLE		MYOKINASE		OLIGO Na^+K^+ATPase		G-3-P OXIDASE	
		-	+	-	+	-	+
H	Spec act	-	0.144(1)	0.01(2)	-	0.025(1)	-
	Tot act	-	25	0.745	-	4.4	-
	% tot act	-	100	100	-	100	-
P1	Spec act	-	0.096(1)	0.0(2)	-	0.0094(1)	-
	Tot act	-	11.2	0	-	1.1	-
	% tot act	-	44.8	0	-	25	-
S1	Spec act	-	0.303(1)	0.015(2)	-	0(3)	-
	Tot act	-	17.7	0.614	-	0	-
	% tot act	-	70.8	82.4	-	0	-
P3	Spec act	-	-	0.0(3)	-	-	-
	Tot act	-	-	0.0	-	-	-
	% tot act	-	-	0.0	-	-	-
P4	Spec act	0.037(2)°	0.10(3)	0.0(3)	-	0.011(1)	-
	Tot act	0.629	1.7	0.0	-	0.25	-
	% tot act	-	6.8	0.0	-	5.7	-

Table 6 (continued)

ENZYME ± Triton X-100 / SAMPLE		HEXOKINASE		G-3-P DH		PG-ISOMERASE	
		-	+	-	+	-	+
H	Spec act	0.262(2)	1.19(3)	0.205(3)	0.347(3)	0.293(3)	1.28(3)
	tot act	54.4	247	42.6	71.8	61	264.8
	% tot act	22.0	100	59.3	100	23.1	100
P1	Spec act	0.373(3)	1.509(2)	0.187(2)	0.318(2)	0.346(1)	0.988(1)
	Tot act	56.8	230.0	28.5	48.5	53	151
	% tot act	23	93.1	39.7	67.5	20.1	57.2
S1	Spec act	0.3	0.445	0.069(2)	0.0724(2)	0.12(2)	0.305(2)
	Tot act	24.7	36.6	5.6	6.0	10	25
	% tot act	10	14.8	7.8	8.4	3.8	9.4
P3	Spec act	0.174(2)	1.33(2)	0.142(2)	0.259(2)	0.292(2)	0.72(2)
	Tot act	24.7	190	20.3	36.7	41.4	102.7
	% tot act	10	76.9	28.3	51.1	15.7	38.9
P4	Spec act	0.385(4)	1.18(3)	0.848(3)	0.316(3)	0.381(3)	1.49(3)
	Tot act	11.1	34	2.5	9.2	11	43
	% tot act	4.5	13.8	2.7	9.9	4.2	16.3

Table 6 (continued)

ENZYME / ±Triton X-100 / SAMPLE		α-GLUCOSIDASE		OU Na^+K^+ATP ase		DHLIP DEHYDROG.	
		-	+	-	+	-	+
H	Spec act	-	0.000149(2)	0.0011(2)	-	0.0065(3)	-
	Tot act	-	0.0205	0.205	-	0.743	-
	% tot act	-	100	100	-	100	-
P1	Spec act	-	0.000154(2)	0.0016(2)	-	0.017	-
	Tot act	-	0.014	0.166	-	0.936	-
	% tot act	-	68.3	81.0	-	126.0	-
S1	Spec act	-	0.00093(2)	0.0(3)	-	0.002(3)	-
	Tot act	-	0.005	0.0	-	0.095	-
	% tot act	-	24.4	0.0	-	12.8	-
P3	Spec act	-	-	0.0021(2)	-	-	-
	Tot act	-	-	0.152	-	-	-
	% tot act	-	-	74.1	-	-	-
P4	Spec act	-	0.000167(2)	0.0049(2)	-	0.03(3)	-
	Tot act	-	0.0027	0.125	-	1.035	-
	% tot act	-	13.2	61.0	-	139.3	-

Fig. 19 Bar charts showing % of total enzyme activity, and specific activities of the enzymes in fractions taken from the plasma membrane purification procedure, in the presence ▨ and absence □ of Triton X-100 0.1% (w/v).

Enzymes are hexokinase (A)

glycerol-3-phosphate dehydrogenase (B),

phosphoglucoisomerase (C),

myokinase (D),

oligomycin sensitive Na^+K^+ATPase (E),

glycerol-3-phosphate oxidase (F).

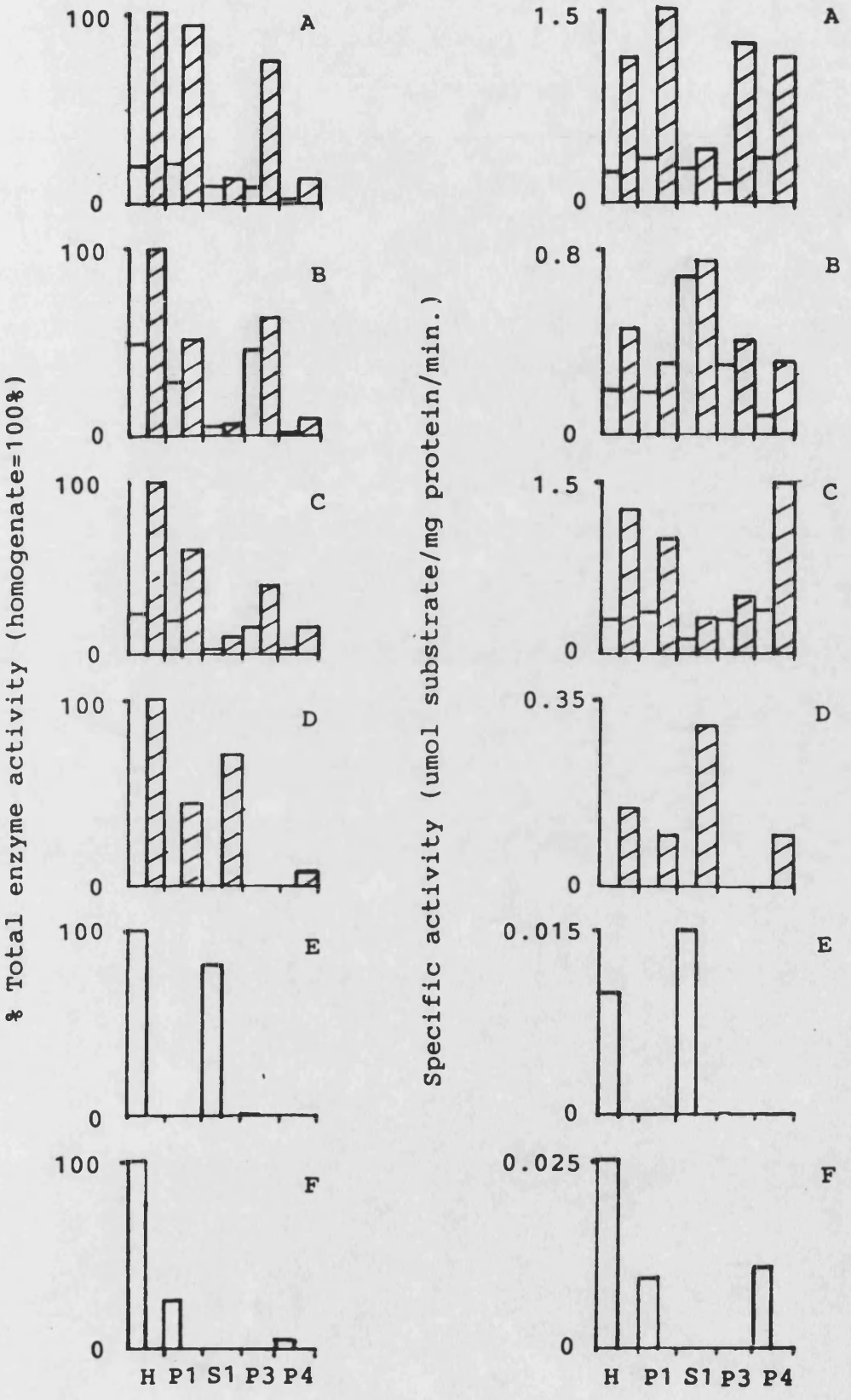
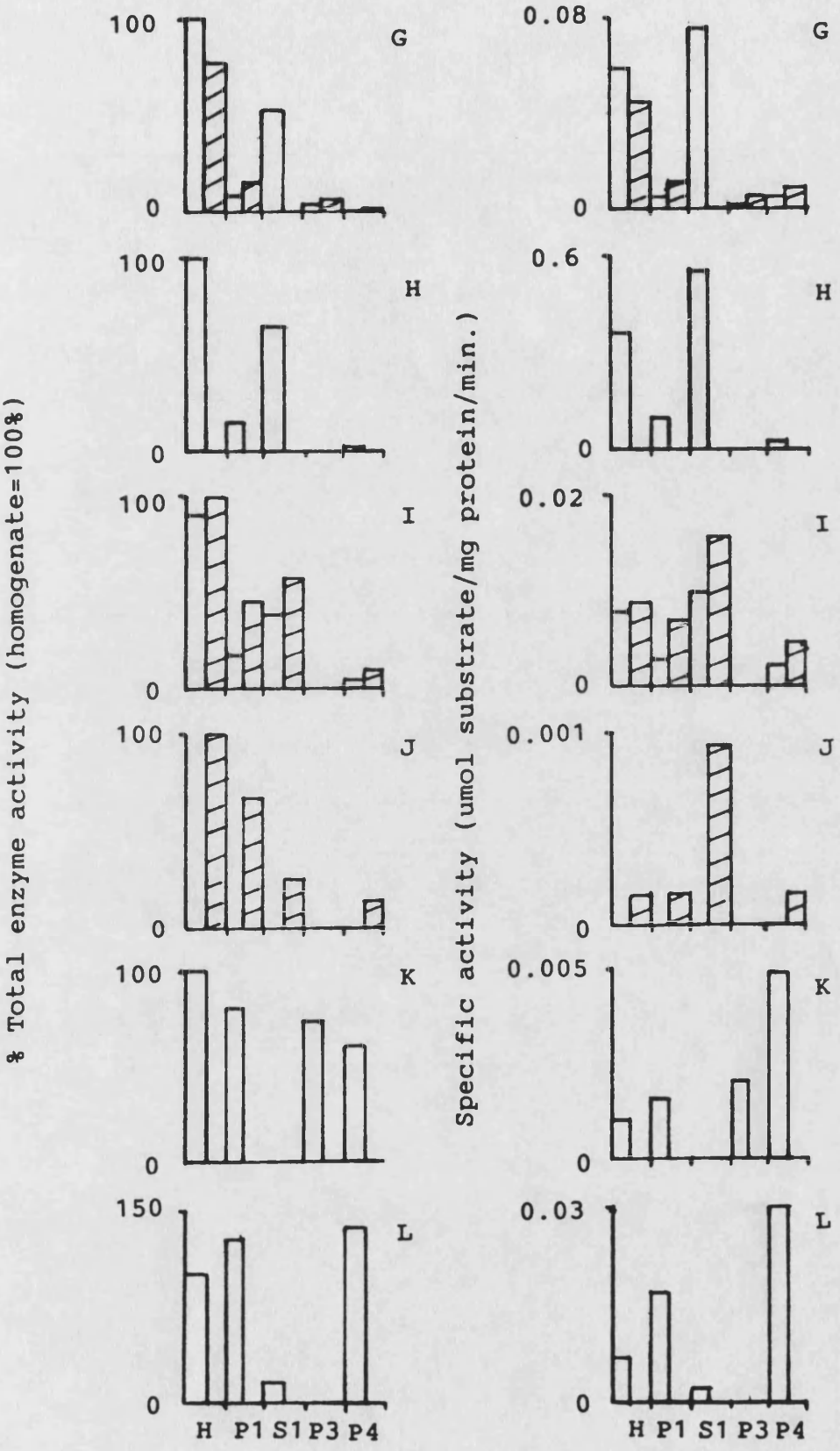

% Total enzyme activity (homogenate=100%)
Specific activity (umol substrate/mg protein/min.)
100
0
A
B
C
D
E
F
1.5
0.8
0.35
0.015
0.025
H P1 S1 P3 P4

Fig. 19 (continued)

Enzymes are pyruvate kinase (G),
malate dehydrogenase (H),
glucose-6-phosphatase (I),
α-glucosidase (J),
ouabain sensitive Na^+K^+ATPase (K),
and dihydrolipoamide dehydrogenase (L).


% Total enzyme activity (homogenate=100%)
Specific activity (umol substrate/mg protein/min.)
100
0
G
100
0
H
100
0
I
100
0
J
100
0
K
150
0
L
H P1 S1 P3 P4
0.08
0
G
0.6
0
H
0.02
0
I
0.001
0
J
0.005
0
K
0.03
0
L
H P1 S1 P3 P4

activity 5.6% of the homogenate value.

Glucose-6-phosphatase (G-6-Pase) and myokinase exhibited similar activity profiles through the plasma membrane purification procedure. In both cases homogenisation released more than 55% of the total enzyme activity into the supernatant S1, with specific activities 1.5 to 2.0 times the homogenate value, in the presence of 0.1% (w/v) Triton X-100. The residual total activity associated with the final plasma membrane pellet P4 was less than 11% of the total homogenate activities for both enzymes, with specific activities 0.55 (G-6-Pase) and 0.7(myokinase) times that of the homogenate values. In the presence of Triton X-100 0.1% (w/v) all the fractions demonstrated latent G-6-Pase activity. This was largest in the pellets P1 and P4 with a more than 2-fold increase in activity on addition of detergent. The measurement of ATP production from 2ADP was used as control experiment to demonstrate true myokinase activity.

The oligomycin sensitive $Na^{+}K^{+}Mg^{2+}$ stimulated ATPase (OL ATPase) and glycerol-3-phosphate oxidase (G-3-P oxidase) demonstrated similar activity profiles through the plasma membrane purification process. G-3-P oxidase was inactivated by 0.1% (w/v) Triton X-100 and for this reason both it, and OL ATPase were not assayed in the presence of detergent.

For both enzymes the pellet P1 contained 25% or less of the total activity found in the homogenate, with specific activities 40% (G-3-P oxidase) and 0%

(OL ATPase) of the homogenate value. OL ATPase was found primarily in the supernatant S1 (82% of the total homogenate activity), however no activity for G-3-P oxidase could be found in the supernatant S1 suggesting possible inactivation of the enzyme with time, once freed from the mitochondrion. No OL ATPase activity was found in the plasma membrane pellet P4, and only 5.7% of the G-3-P oxidase, of a specific activity under half the value for the homogenate.

Hexokinase, glycerol-3-phosphate dehydrogenase (G-3-PDH) and phosphoglucoisomerase (PG-isomerase) exhibited latent activity in all fractions through the purification procedure. In the cases of hexokinase and PG-isomerase in pelletsP1 and P4, Triton X-100 (0.1% (w/v)) induced an increase in activity of 3 to 4.6-fold. Significantly, for all three enzymes, greater than 51% of the total activity stayed with the plasma membrane pellet P1, and release of enzyme activity into the supernatant S1 did not exceed 14.8% of the total homogenate value.

The specific activities of all three enzymes in the plasma membrane pellets P1, P3 and P4 remained approximately the same as that of the homogenate value throughout the purification procedure. The high total enzyme activities (retained in the plasma membrane pellets P1 and P3) were, however, reduced significantly by the sucrose density gradient centrifugation step. This resulted in less than 17% of the total activity of any of the three enzymes being retained in the final plasma membrane pellet, P4.

α -glucosidase activity was extremely low in all fractions and required an incubation time of 24h to allow accurate measurements to be taken. Sixty-eight percent of the total homogenate activity was retained in the plasma membrane pellet P1 at a specific activity equivalent to that of the homogenate. Under 25% of the total activity was released into the supernatant S1. DNAase treatment and sucrose density gradient centrifugation decreased the total amount of enzyme activity in pellet P4 to 13.2% of that of the homogenate, but with a specific activity 12.1% higher than the homogenate value.

The ouabain sensitive Na^+K^+ATPase (OU ATPase) and dihydrolipoamide dehydrogenase (DHLIP DH) co-purified with the plasma membranes. DHLIP DH demonstrated significant activation, without detergents, through the plasma membrane isolation procedure. Eighty-one percent of the total OU ATPase activity was retained in pellet P1, and 126% of the total DHLIP DH activity. No detectable OU ATPase was found in the supernatant S1 and DHLIP DH activity was 12.8% of the total, at a specific activity less than 1/3 that of the homogenate.

The retention of enzyme activity in pellet P1 was mirrored by an increase in specific activity of 1.45 (OU ATPase) and 2.6 (DHLIP DH) times the homogenate value. In the final plasma membrane pellet P4 61% of the total OU ATPase and 139% of the total DHLIP DH activity were retained, with specific activities greater than 4.4-fold that of the homogenate value.

It should be noted that the activities quoted for the OU ATPase and OL ATPase represent the "best"

values obtained. Reproducibility and inhibition attributable to oligomycin and ouabain were extremely small compared to the background values, particularly in the more dilute homogenate and supernatant S1 samples.

Alteration of the method of Post and Sen (1967) to include stabilisation of colour by addition of arsenite/ citrate solution as outlined for the G-6-Pase assay, did not improve the accuracy or reproducibility of the OL ATPase or OU ATPase.

Figure 20 is an electron micrograph of the final purified plasma membrane (x 50,000) demonstrating the cross-section of the flagella and pellicular microtubule array attached to the plasma membrane. These structures are similar to those shown by Voorheis et al. (1979) which are definitive proof of plasma membranes.

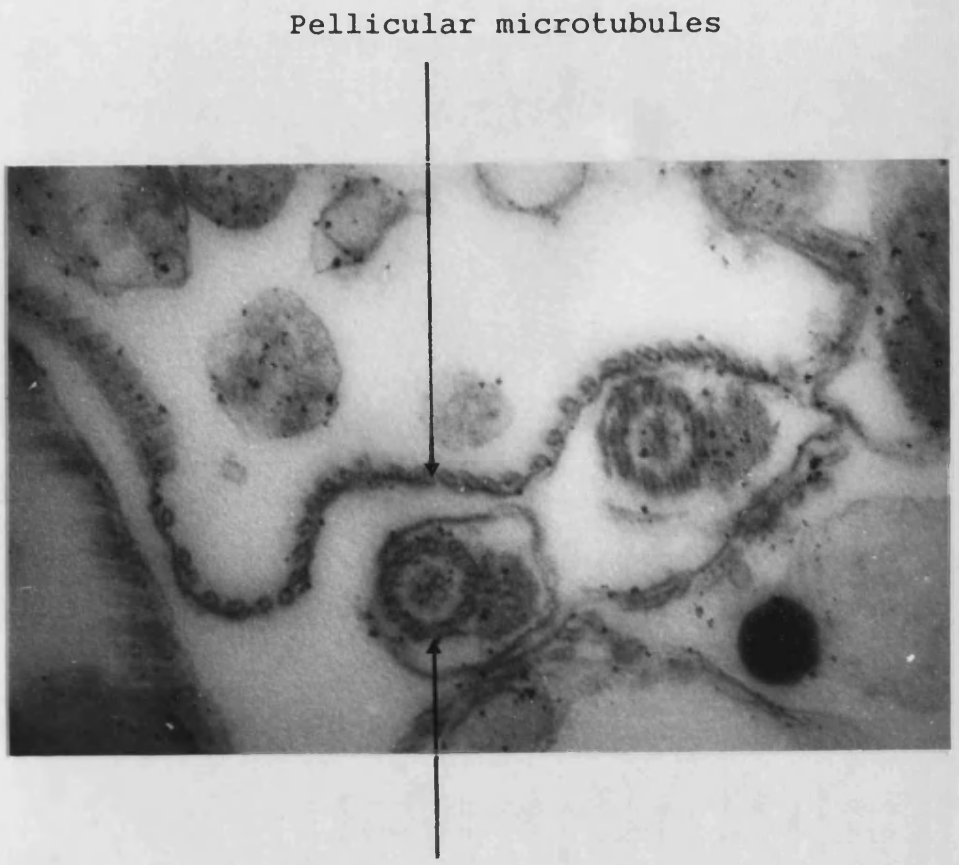


Fig. 20 Electron micrograph (x50,000) of purified trypanosome plasma membranes demonstrating the pellicular microtubules and flagella cross section.

2.2 DETERMINATION OF THE PHOSPHATE DONOR SPECIFICITY AND THE Km AND Vmax FOR THE PLASMA MEMBRANE HEXOKINASE

Table 7 summarises the phosphate donors and combinations of phosphate donors tested on the plasma membrane and supernatant hexokinases in the presence and absence of magnesium ions (5mM) and phenylarsine oxide (0.2mM). In the presence of Mg^{2+} ions both ATP and ADP donated a phosphate group to D-glucose. In the absence of Mg^{2+} phosphorylation of D-glucose by ADP did not occur and phosphorylation by ATP was reduced to less than 10% of the value in the presence of Mg^{2+}.Phosphoenol pyruvate (PEP), inorganic phosphate (Pi), pyrophosphate (PPi) and D-glucose-6-phosphate(G-6-P) did not donate phosphate to D-glucose unless used in combination with ADP or ATP. The hexokinase activity in supernatant S1 demonstrated the same properties as those in the plasma membrane fraction.

Fig.21A and Fig. 22A show the production of D-glucose-6-phosphate with increasing D-glucose concentration for ATP and ADP respectively. The Km and Vmax for D-glucose phosphorylation in the presence of ATP and ADP were determined using the direct linear plot of Cornish-Bowden and Eisenthal (1974), Fig. 21B and 22B. The Km and Vmax for D-glucose with ATP as phosphate donor were 48μmolar and 49.4nmol G-6-P/mg protein/min respectively, and for ADP as phosphate donor, 139μmolar and 21.6nmol G-6-P/mg protein/min respectively. The difference in

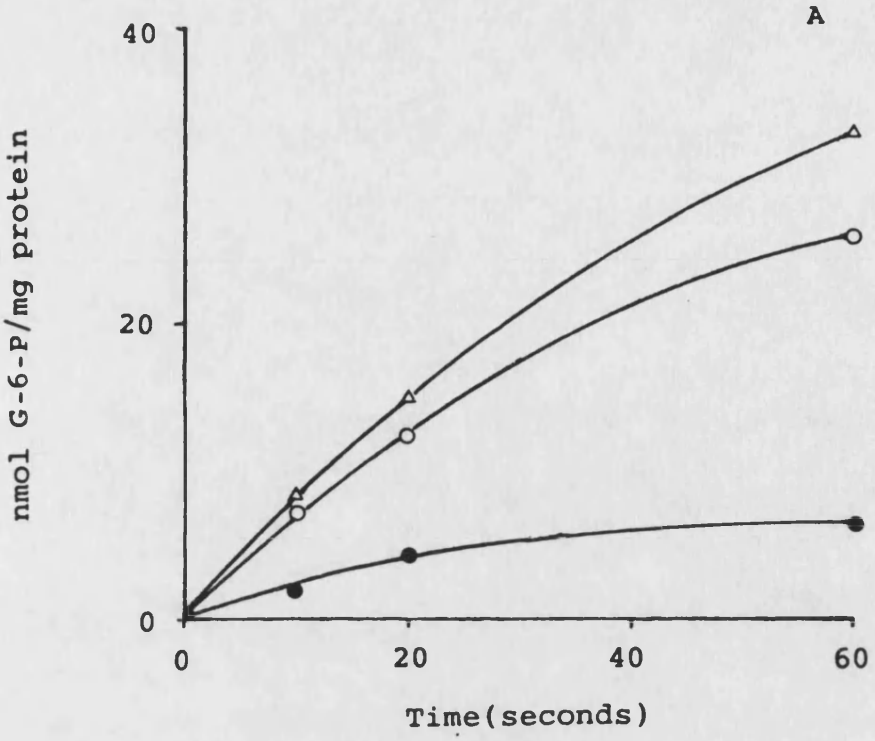
Table 7. Combinations of possible phosphate donors to determine phosphate donor specificity of plasma membrane associated hexokinase (type) activity.

Phosphate donor / Fraction	Mg^{2+}	ATP	ATP Mg^{2+}	PEP	PEP Mg^{2+}	ADP PEP	ADP PEP Mg^{2+}	ADP	ADP Mg^{2+}	Pi	Pi Mg^{2+}	G6P Mg^{2+}	PPi Mg^{2+}
Supt. S1	-	+	+	-	-	-	+	-	+	-	-	-	-
Plasma membrane pellet P4	-	+	+	-	-	-	+	-	+	-	-	-	-

+ denotes production of D-glucose-6-phosphate

- denotes no production of D-glucose-6-phosphate

D-glucose was present in all assays at a final concentration of 1mM.



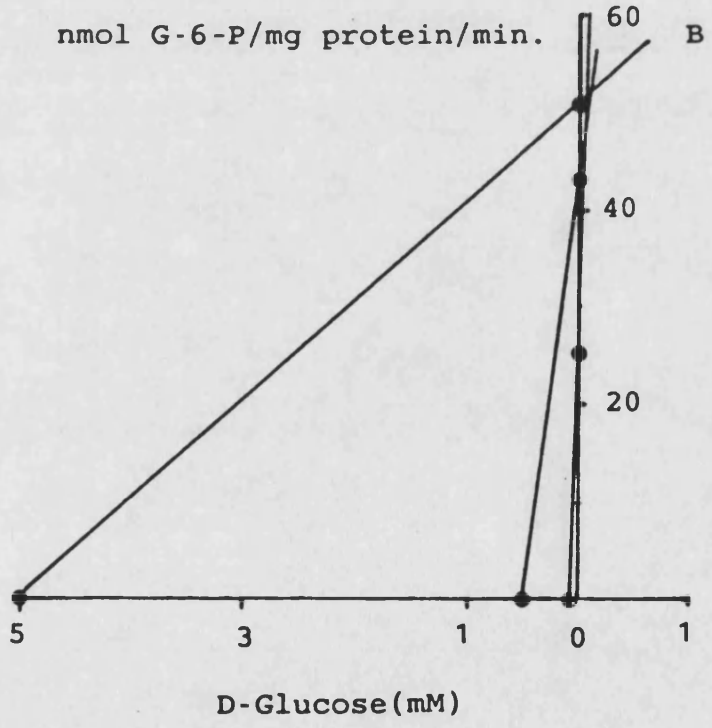
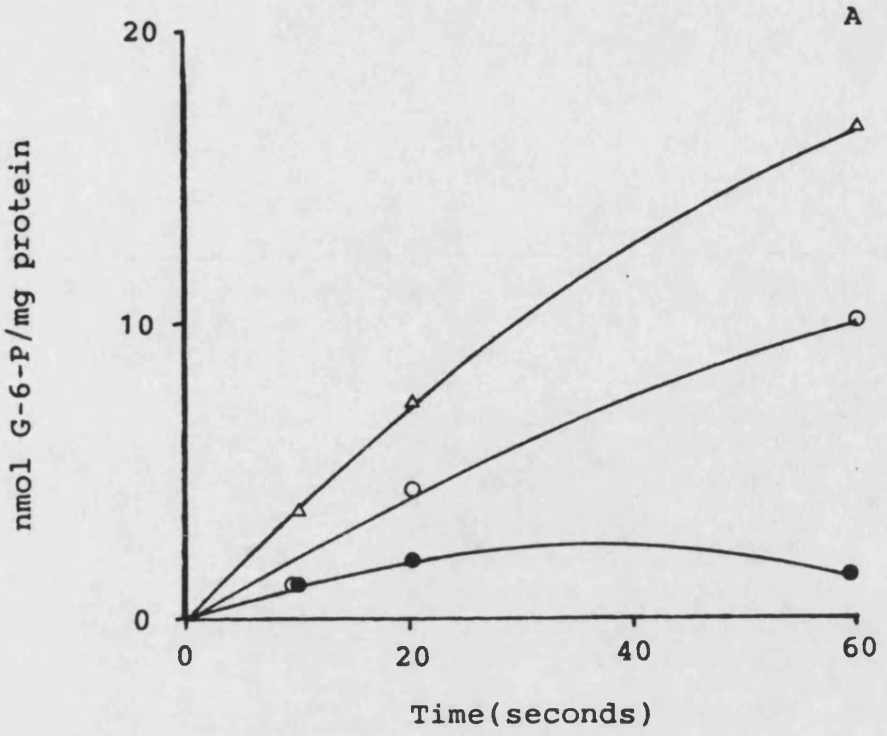


Fig.21A. nmol of G6P produced by plasma membrane hexokinase against time at 0.05mM(●),0.5mM(○) and 5.0mM(△)D-glucose, and Fig.21B, the direct linear plot of the data to determine Km and Vmax for D-glucose. ATP = phosphate donor.



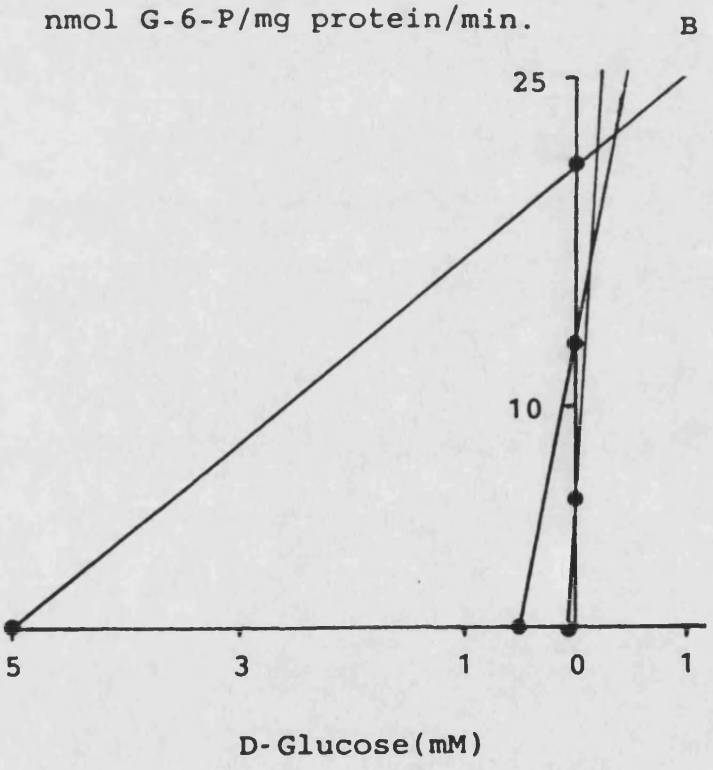


Fig.22A, Time course of G6P produced by plasma membrane hexokinase at 0.05mM(●),0.5mM(○) and 5.0mM(△)D-glucose, and Fig.22B, the direct linear plot of the data to determine Km and Vmax for D-glucose. ADP = phosphate donor.

kinetic constants using ATP or ADP as the phosphate donor were likely to be due to the presence of myokinase in the plasma membrane preparation. This point is discussed further in the Discussion section 2.0.

The accuracy of the diethyl-ether extraction technique was tested by replacing trypanosome protein with an equal quantity of bovine serum albumin, and ATP and D-glucose by D-glucose-6-phosphate. The assayed D-glucose-6-phosphate agreed within 10% of the initial starting value.

2.3 RELEASE OF DIHYDROLIPOAMIDE DEHYDROGENASE FROM PLASMA MEMBRANES AND RECONSTITUTION INTO PHOSPHOLIPID LIPOSOMES

Dihydrolipoamide dehydrogenase co-purified with the plasma membranes such that 139% of the homogenate total activity was recovered in the plasma membrane pellet P4.

Plasma membranes treated with increasing concentrations of potassium chloride up to 1.0M did not release more than 13.4% of the total DHLIP DH activity into the supernatant. This was mirrored by a retention of DHLIP DH activity in the pellets after treatment with increasing concentrations of KCl, fig. 23B.

Treatment of the plasma membranes with increasing Triton X-100 concentrations released DHLIP DH activity into the supernatant at concentrations greater than 0.05% (w/v) Triton X-100. Release of the DHLIP DH increased

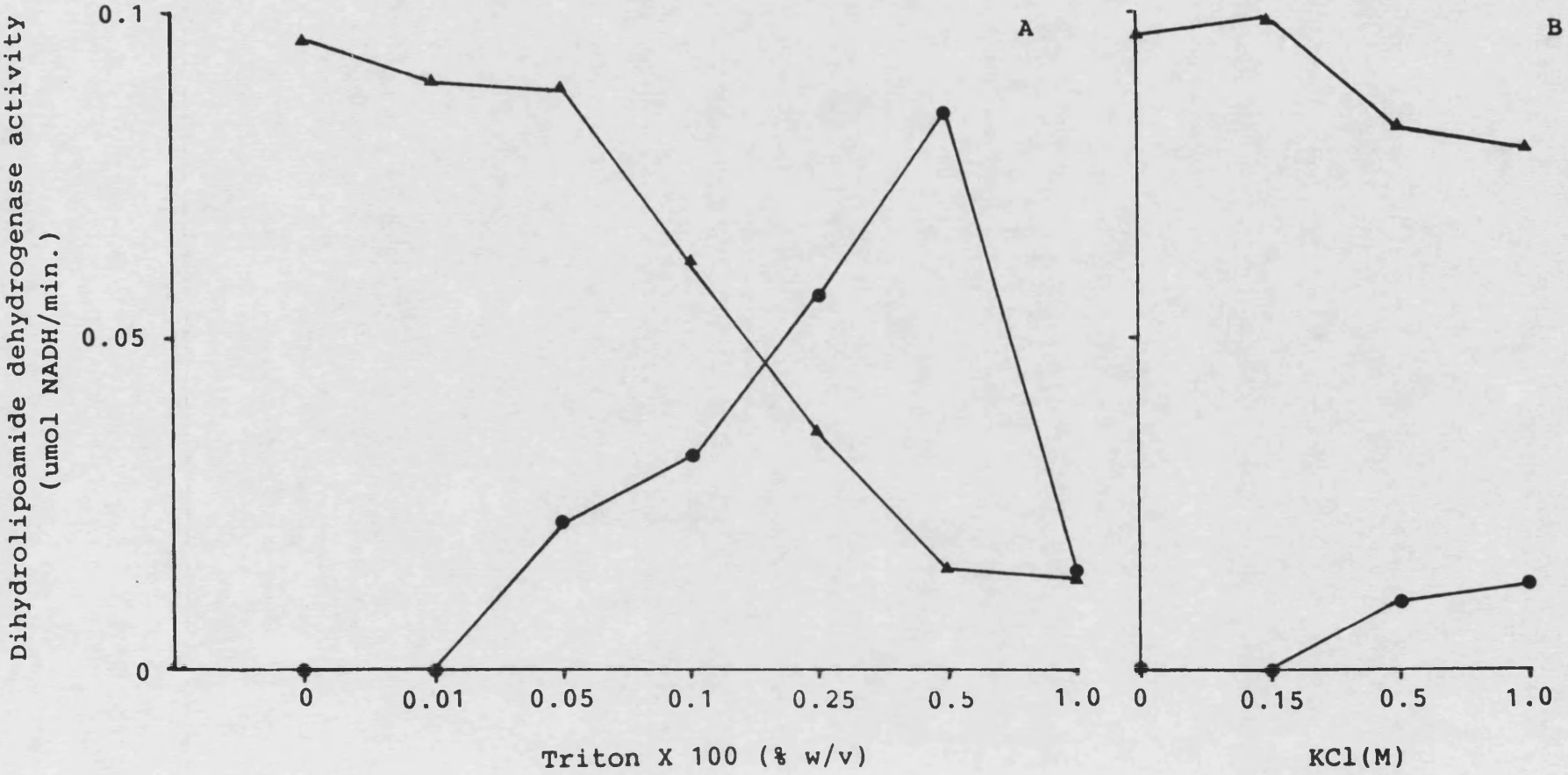


Fig. 23 Total dihydrolipoamide dehydrogenase activity in the extracts (●) and pellets (▲) of plasma membranes treated with Triton X-100 (A) and potassium chloride (B).

with increasing Triton X-100 concentration, whilst activity in the post extraction pellets decreased with increasing detergent concentration, fig. 23A.

A peak of extraction as measured by total enzyme activity released into the supernatant was reached at 0.5% (w/v) Triton X-100 at which over 80% of the total DHLIP DH activity (compared to the 0 Triton X-100 sample) was recovered in the supernatant. At 1.0%(w/v) Triton X-100 the activity found in the supernatant decreased dramatically, whilst retaining the activity in the post-extraction pellet. This may be caused by inactivation of the enzyme at high detergent concentrations in aqueous solutions.

The optimum Triton X-100 extract of the dihydrolipoamide dehydrogenase activity (0.5%(w/v)Triton X-100) was reconstituted into phosphatidyl choline vesicles by the freeze/thaw sonication method. Fifteen percent of the total plasma membrane dihydrolipoamide dehydrogenase (21% of the homogenate value) was recovered in the proteoliposome pellet with a specific activity of 0.037μmol NADH/mg protein/min, a figure 23% higher than the specific activity of the plasma membranes pellet P4 (specific activity 0.03μmol NADH/mg protein/min.

The proteoliposomes containing dihydrolipoamide dehydrogenase reconstituted from the 0.5% (w/v) Triton X-100 extract retained 63% of the total dihydrolipoamide dehydrogenase activity after dilution (1:5), sonication, freezing and thawing, repeat sonication and further dilution, before collection by ultracentrifugation.

3.0 EFFECT OF ALKALI TREATMENT ON TRYPANOSOME PLASMA MEMBRANES

Increasing concentrations of sodium hydroxide (5, 10 and 15mM) resulted in increased disruption of the plasma membrane structure, such that the size of particulate matter observed under light microscopy (x400) decreased with increasing alkali. Total membrane protein recovered by ultracentrifugation after treatment ranged from 14% (15mM NaOH) to 40% (5mM NaOH) of the total protein used, fig. 24. After undergoing the freeze/thaw vesiculation procedure total recoverable protein remained at 14% for 15mM NaOH treated membranes, but decreased for the lower concentrations of sodium hydroxide treatment to 19% of the total for 5mM NaOH, and 15.4% of the total for 10mM NaOH treated membranes.

Dihydrolipoamide dehydrogenase activity, could not be measured in the 10mM and 15mM NaOH treated membranes and only 1.0% of the total initial activity could be found in the 5mM NaOH treated membranes.

Infinite-trans D-glucose transport assays produced no D-glucose influx into the membranes preincubated with 100mM D-glucose/10mM sodium phosphate buffer at room temperature, suggesting there were no vesicle bodies present.

The frozen and thawed alkali extracted membranes did demonstrate D-glucose influx with time; however fast initial filling of the vesicles followed by a decrease to an equilibrium concentration did not occur. Influx was gradual suggesting a non-facilitated process, fig.25.

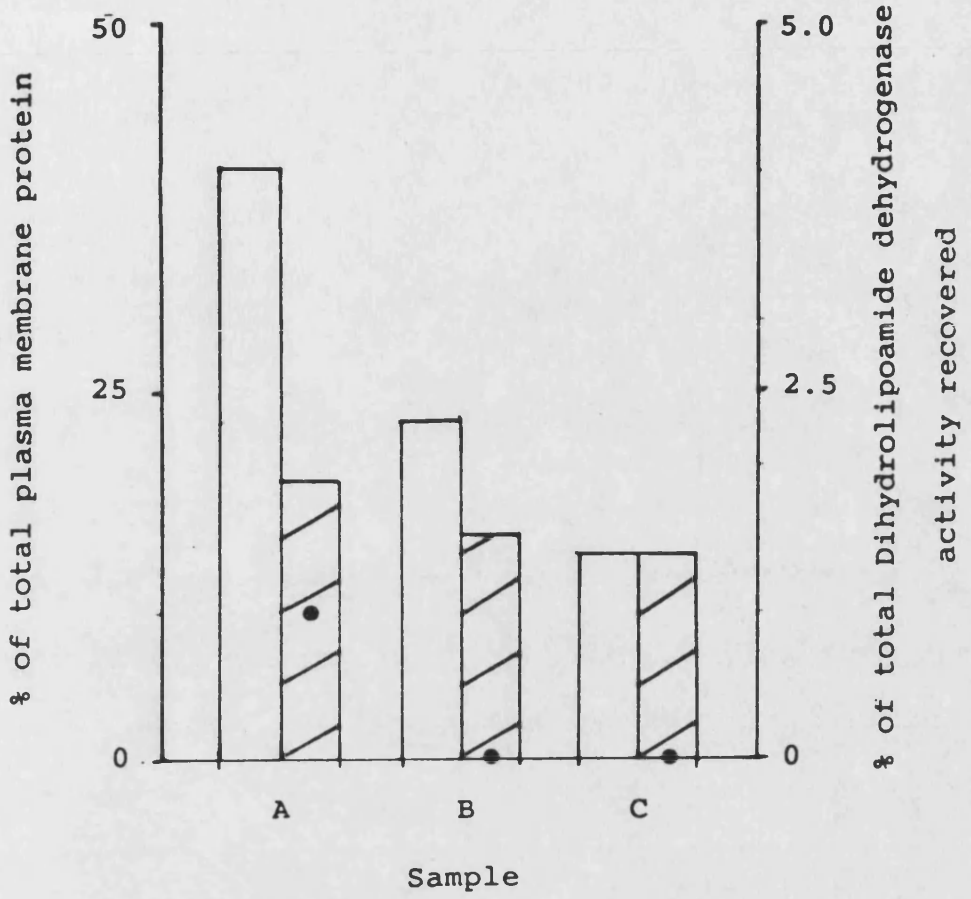


Fig. 24. Bar chart showing total plasma membrane protein retained after alkali treatment(□)and freeze/thaw treatment of alkali treated membranes(▨) at 5mM NaOH (A), 10mM NaOH (B) and 15mM NaOH (C), including % total dihydrolipoamide dehydrogenase retained in final alkali and freeze/thaw treated membranes (●).

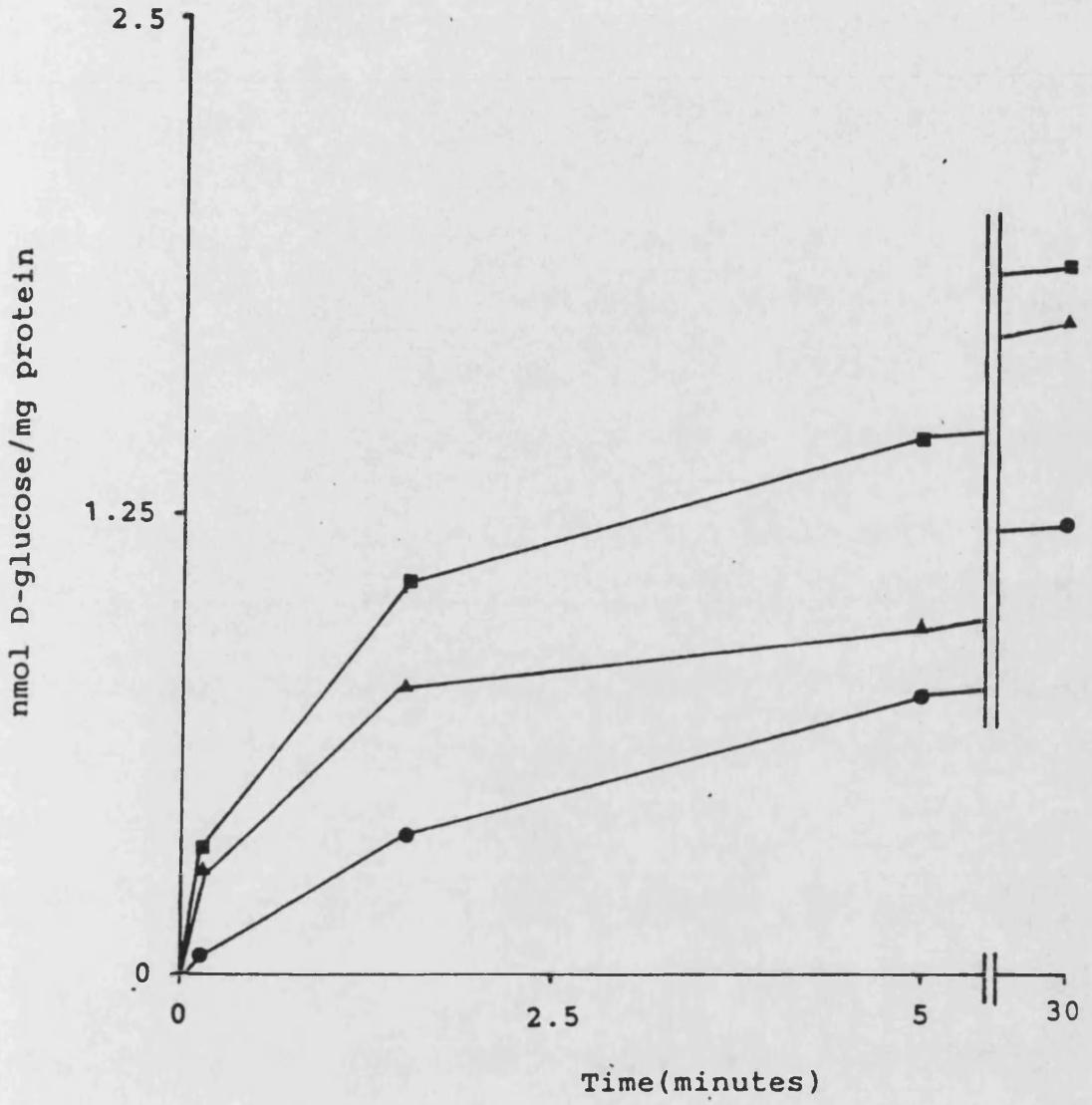


Fig. 25. Time course of infinite-trans D-glucose influx into vesicles from alkali (5mM (●), 10mM (■) and 15mM (▲) sodium hydroxide) and freeze/thaw treated plasma membranes. All values ± SE no greater than 11% of the quoted value.

3.1 CALCIUM ION TREATMENT TO RELEASE THE PLASMA MEMBRANE PELLICULAR ARRAY, AND D-GLUCOSE TRANSPORT IN FREEZE/THAW VESICLES FROM THE PROCESS

Trypanosome plasma membranes treated without calcium ions as a control experiment in the pellicular depolymerisation process, released 27% of the total protein into the supernatant after collection of the plasma membranes at 9,000g, bar chart fig. 26.

During the incubation of the membranes at 37°C the plasma membranes aggregated into large bodies, with significant observable small particles in the background. Defatted bovine serum albumin 1%(w/v) was added to the depolymerisation solutions to prevent aggregation (Voorheis, personal communication) by binding to fatty acid residues on the plasma membrane surface after release of the protein coat. The addition of BSA however did not prevent the plasma membrane aggregation. Microscopic examination showed the aggregated membranes and background small particles, as noted for the process in the absence of BSA. Twenty-four percent of the plasma membrane protein was released into the supernatant, as measured after collection of the plasma membranes at 9,000g. The release of protein into the supernatant was mirrored by the release of 19.5% of the total DHLIP DH activity into the supernatant, with or without BSA.

Addition of calcium chloride to samples incubated with and without BSA at 0.1, 0.5 and 1.0mM calcium chloride increased the total protein released into the

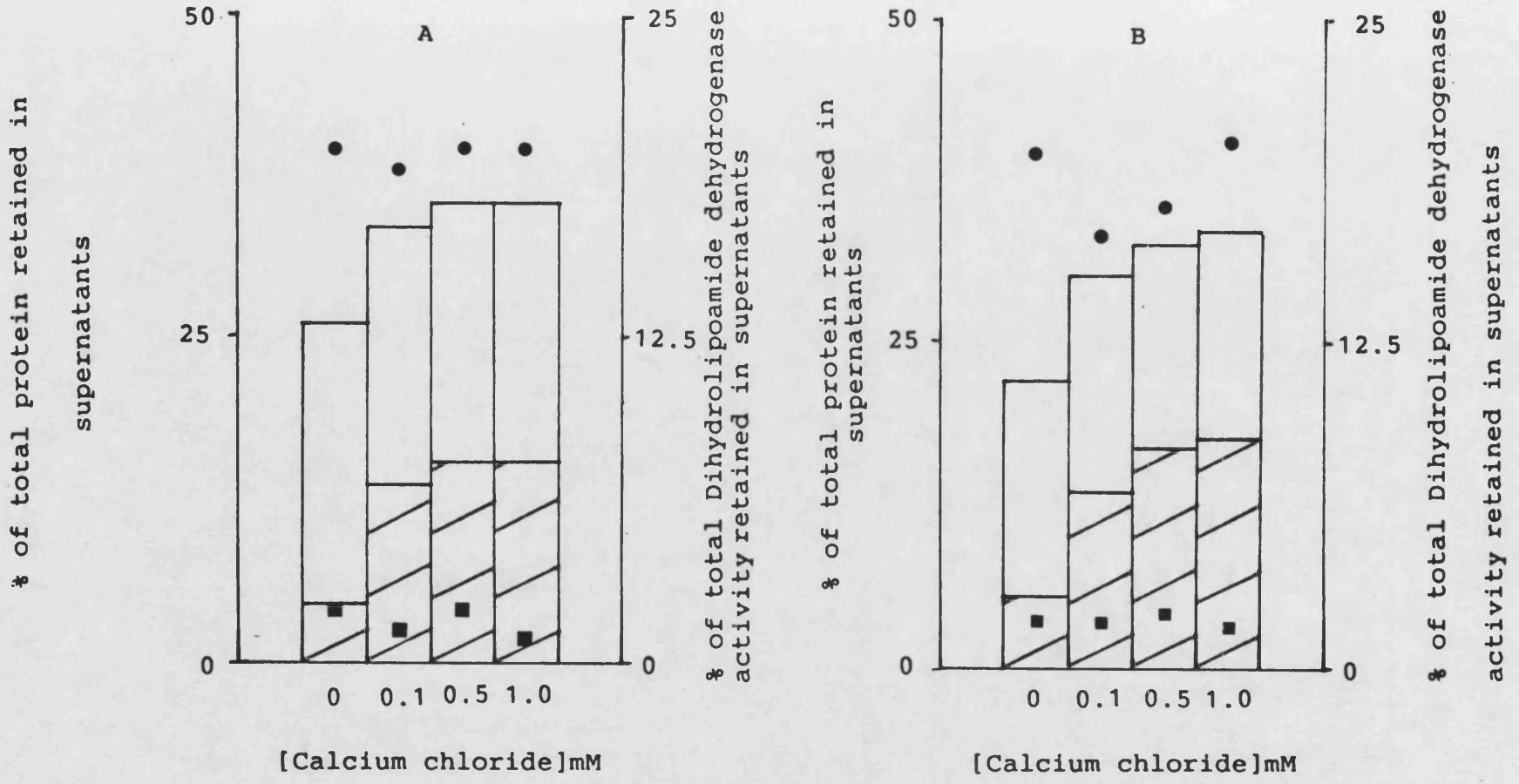


Fig. 26. Bar chart showing % of total plasma membrane protein in supernatants against increasing calcium chloride concentration in the presence(B) and absence(A)of 1%(w/v)defatted BSA, after centrifugation at 9,000g,10min (□) and 20,000g,60min(▨). The % of the total plasma membrane dihydrolipoamide dehydrogenase activity retained in the supernatants after centrifugation 9,000g, 10min(●) and 20,000g, 60min(■) under the same conditions are also included.

supernatant, figs. 26 A and B, such that at 0.1mM Ca^{2+} (no BSA) 33.4% and 0.1mM Ca^{2+} (BSA present), 30% of the total protein was found in the supernatant fraction. These figures rose slightly with increasing Ca^{2+} concentration to reach 35.5% and 33.6% of the total protein at 1.0mM Ca^{2+}, without and with BSA respectively. In all cases the protein released into the supernatant contained dihydrolipoamide dehydrogenase activity of between 16.8 and 20.3% of the total, fig. 26A and B.

Each supernatant when centrifuged at 20,000g yielded a further small pellet of material. Dihydrolipoamide dehydrogenase activity decreased to between 1.5 and 2.6% of the total original activity, fig. 26A and B, and a residual protein content in the supernatant of between 4.5% (no calcium or BSA present) to 17.6% (1.0mM Ca^{2+}, 1% (w/v) BSA) of the total starting protein, fig. 26A and B.

The maximum protein release occurred for 1.0mM Ca^{2+} in the presence of 1% BSA (w/v) where subtraction of the final calcium free value in the presence of 1% BSA (w/v) yielded a net protein release of 12.3% of the total. Calcium ion concentrations above 0.1mM in the presence and absence of BSA did not release a significantly greater quantity of protein into the supernatants.

Fig. 27 shows the influx of D-glucose into freeze/thaw produced vesicles made from the 0.1mM Ca^{2+} treated plasma membrane pellet, preloaded with 100mM D-glucose. The figure demonstrates a leakage of D-glucose into the vesicles, and not the accumulation of radiolabelled

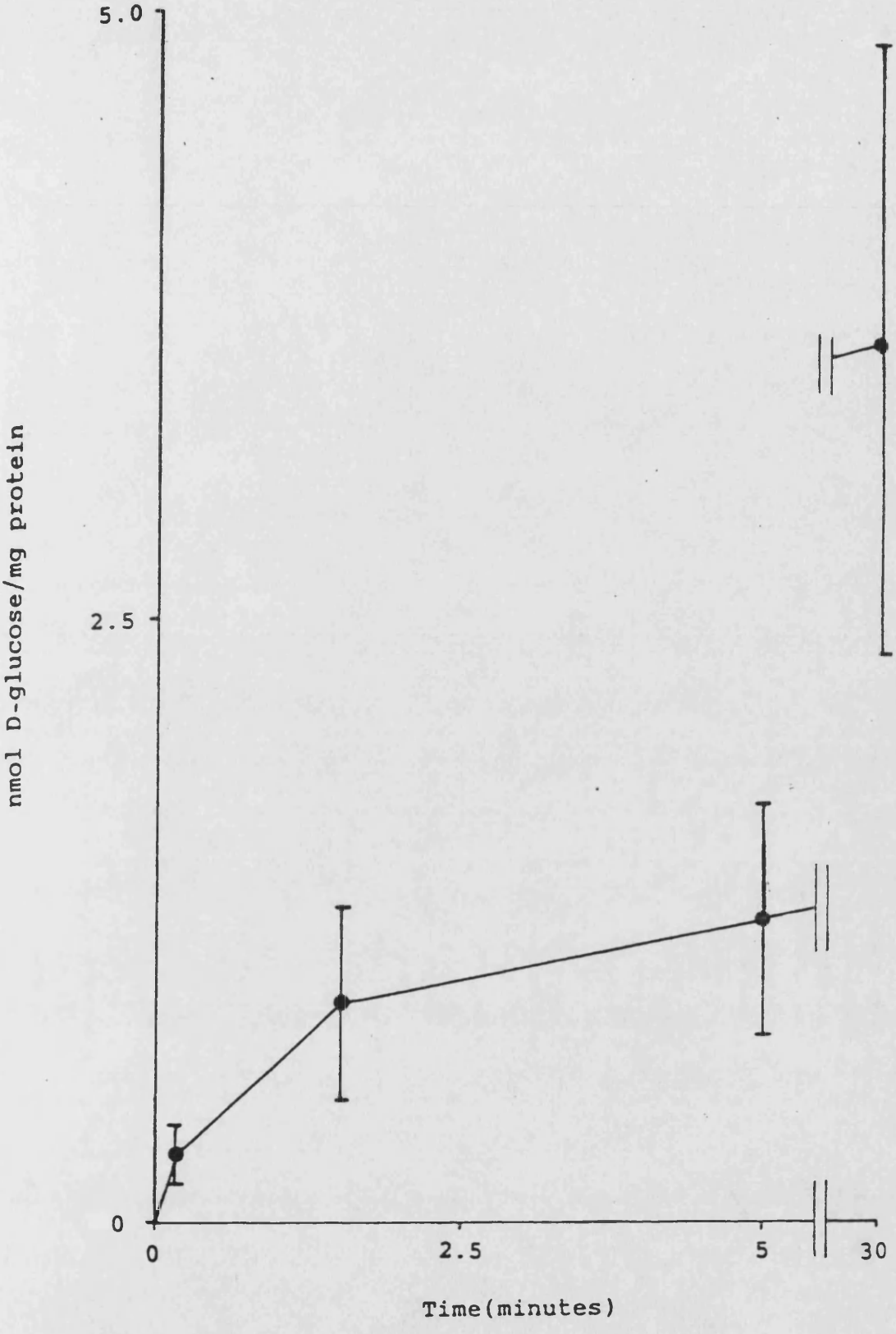


Fig. 27. Infinite-trans D-glucose influx into vesicles prepared by freezing and thawing of the 0.1mM Ca^{2+} treated plasma membranes.

D-glucose above the equilibrium value expected for a facilitated process using an infinite-trans protocol.

4.0 EFFECT OF INCREASING OSMOLARITY ON THE PRODUCTION OF PLASMA MEMBRANE VESICLES AND D-GLUCOSE TRANSPORT IN THOSE VESICLES

Trypanosome plasma membranes were severely disrupted by the rapid freezing and thawing process yielding two populations. Firstly a population of small particles visible as background under light microscopy, and secondly a population of large pieces of residual plasma membrane material, clearly seen under light microscopy as aggregates.

Initial freezing and thawing of the plasma membranes and subsequent freezing and thawing of each large debris pellet, released a decreasing amount of protein into the respective supernatants. The range of phosphate buffer concentrations (5, 10, 30 and 100mM) used in the freezing and thawing process tested the effect of increasing osmolarity and salt concentration on the vesiculation process.

Table 8 summarises the total protein used at each phosphate concentration, the total protein recovered in each supernatant (S1 to S5), the sum of the supernatant total protein values (S_T), and percentage of the total protein they represent, the total protein recovered in the final plasma membrane vesicle pellet (P_F) and the percentage this value represents of the initial total protein.

Total protein released into the supernatants (S_T) was between 26.8% (100mM NaPi) and 30.2% (5mM NaPi) of

Table 8. Summary of the total protein (mg) and % total protein found in fractions taken from the freeze/thaw procedure at 5,10,30 and 100mM sodium phosphate buffer.

Sodium phosphate buffer (mM) / Fraction	5	10	30	100
Plasma membranes	25.1	24.75	24.9	24.6
Supernatant S1	4.1	3.3	3.4	2.9
Supernatant S2	1.6	1.6	1.5	1.3
Supernatant S3	0.9	0.9	1.0	1.1
Supernatant S4	0.7	0.6	0.7	0.7
Supernatant S5	0.3	0.4	0.5	0.6
Supernatant total S_T	7.6	6.8	7.0	6.6
% total protein in S_T	30.2	27.5	28.1	26.8
Vesicle total protein P_F	2.1	2.3	2.0	2.0
%total protein in P_F	8.4	9.3	8.0	8.1

the initial total protein, and in the final pellet P_F, between 8.0% (30mM NaPi) and 9.3% (10mM NaPi) of the total protein. In subsequent experiments using 10mM sodium phosphate buffer, the percentage yield of plasma membrane vesicles in terms of total protein was 9.7%±1.5%, and the yield of dihydrolipoamide dehydrogenase in terms of total activity was 8.4%±3.1%.

Fig. 28 compares the protein banding patterns of whole cells, plasma membranes and the plasma membrane vesicles (10mM NaPi buffer) on SDS-PAGE 5% to 20% gels. The most significant aspect of the patterns is the decrease in the intensity of the band at 57.5K, and the increase in the intensity of the protein bands at 42.5K and 40K. The 57.5K protein band approximates to the 55K band of Dolan et al. (1986), corresponding to tubulin found in the plasma membrane pellicular microtubules, suggesting that the freeze/thaw procedure is releasing plasma membranes from the cytoskeleton.

D-glucose infinite-trans counterflow transport into the vesicles at each phosphate buffer concentration, with time, are shown in fig. 29. Extravesicular D-glucose concentration was 1mM. All the vesicles regardless of buffer concentration demonstrate an accumulation of radiolabelled D-glucose above the equilibrium value with time. This effect could be negated by increasing the extravesicular D-glucose concentration to 100mM, thus saturating the D-glucose transporter giving the gradual influx of radiolabelled D-glucose as shown for 10mM NaPi and 100mM NaPi vesicles, fig. 29. These results

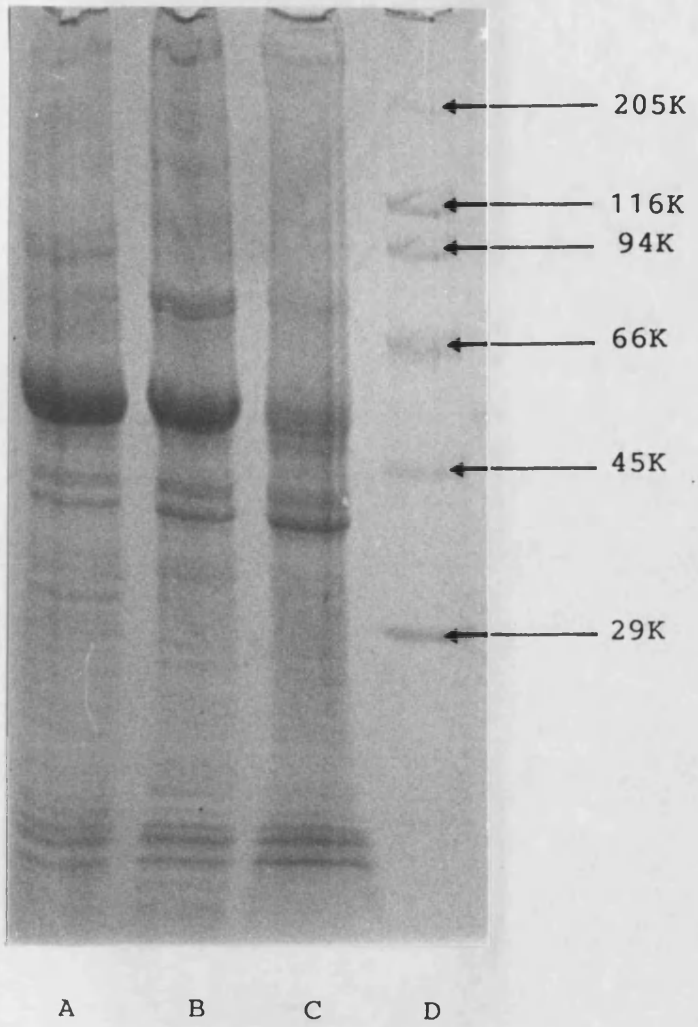


Fig. 28 SDS-PAGE gel 5 to 20% acrylamide showing protein bands from whole trypanosomes (A), plasma membrane (B) and plasma membrane vesicles (C). Lane D is the molecular weight markers, with molecular weights as indicated.

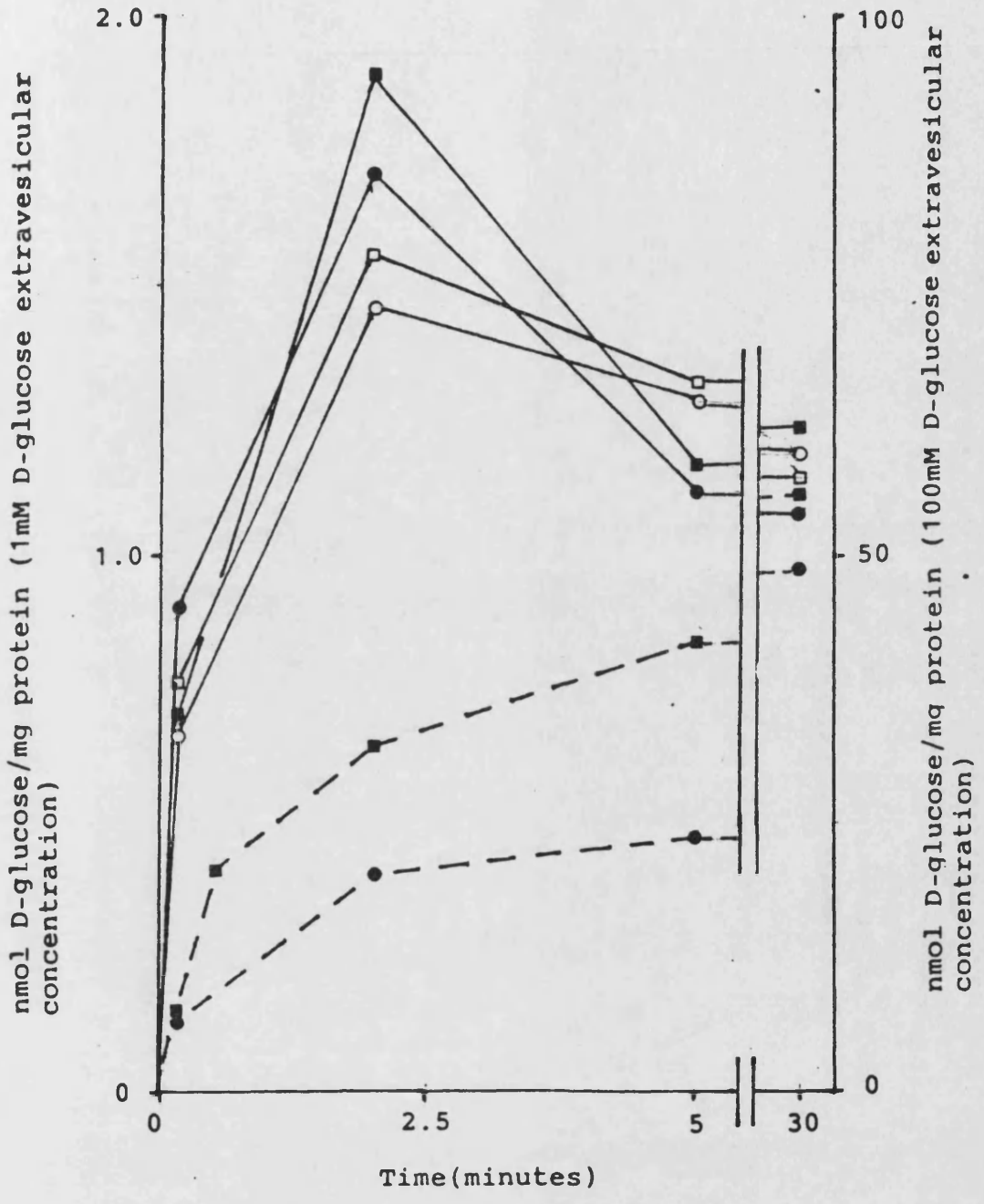


Fig. 29. Infinite-trans D-glucose influx into vesicles preloaded with 100mM D-glucose prepared in 5mM(○), 10mM(●), 30mM(□), and 100mM(■) sodium phosphate buffer, at 1mM extravesicular D-glucose (——) and 100mM extravesicular D-glucose(----). All values ± SE of 16% or less.

suggest a facilitative transport system for D-glucose is present.

Maximum D-glucose accumulated in the vesicles at 2min with 1mM extravesicular D-glucose concentration was 1.89nmoles/mg protein (100mM NaPi buffer) and the least 1.45nmoles D-glucose/mg protein at 5mM NaPi buffer. The accumulation of D-glucose over the first 10 seconds of incubation was 0.9nmoles/mg protein for 10mM sodium phosphate buffered vesicles and the least 0.66nmoles of D-glucose/mg protein for 30mM NaPi buffered vesicles.

5.0 RECONSTITUTION OF TRYPANOSOME AND ERYTHROCYTE PLASMA MEMBRANE PROTEINS

The results from the reconstitution studies have been categorised according to the D-glucose transport assay used, *i.e.* Zero-trans influx or infinite-trans (counterflow) influx. These are then subdivided into the type of reconstitution process utilised.

5.1 ZERO-TRANS D-GLUCOSE INFLUX INTO RECONSTITUTED PROTEOLIPOSOMES

These studies utilised proteoliposomes with no intravesicular D-glucose preloading, relying on the measurement of the filling of the proteoliposomes and the kinetics of the process to determine if facilitated transport occurred.

5.1.1 Reconstitution by the freeze/thaw/sonication procedure

The percentage of the total protein extracted from human erythrocyte "ghosts" and trypanosome plasma membranes by Zwittergent 14 (2 and 3mM), Triton X-100 (0.5% (w/v)) and n-octyl-β-D-glucopyranoside (50mM) are summarised in the table below.

Membrane source / Detergent	Trypanosome	Erythrocyte
Zwittergent 14 (2mM)	28-35	43.5
Zwittergent 14 (3mM)	32-45	47
Triton X-100 (0.5%(w/v))	22-38	35-42
n-octyl-B-D-glucopyranoside (50mM)	34	41

For both erythrocyte "ghosts" and trypanosome plasma membranes Zwittergent 14 extracted the most total protein. The solubilisation of the erythrocyte "ghosts" protein by Triton X-100 and n-octyl-β-D-glucopyranoside was significantly lower than that stated by Kasahara and Hinkle (1976) in which Triton X-100 (0.5%(w/v)solubilised 70% of the available protein and n-octyl-β-D-glucopyranoside 56%. This discrepancy may well be a result of the gentle treatment of the membranes during extraction, *ie.* only inverting the tubes to mix.

Biobeads SM2 were found to be efficient in removing Zwittergent 14 and Triton X-100, but resulted in a loss of protein of between 6 to 15% of the total extracted protein. Dialysis of n-octyl-β-D-glucopyranoside extracts yielded no measurable loss of protein.

In assaying the proteoliposomes produced by this technique it was found that the most reproducible results were obtained if the lipid used in the reconstitution was sonicated to clarity before use. This process could be hastened if the lipid was vortex mixed for 10 minutes before sonication.

Proteoliposomes formed from the well sonicated lipid entered the 0.22μm millipore filters within 5 seconds of application, allowing fast and efficient washing of the trapped proteoliposomes. Use of liposomes not sonicated to clarity resulted in blocked filters, increasing washing and filtering times dramatically, or in the worst cases prevented filtration and washing totally.

D-glucose concentrations of 0.1mM to 10mM for erythrocyte derived proteoliposomes, and 0.1mM to 20mM for trypanosome plasma membrane derived proteoliposomes were used to highlight the influx of D-glucose attributable to facilitated transport rather than leakage down the D-glucose gradient.

Fig. 30 shows the influx of 0.1mM D-glucose into proteoliposomes derived from erythrocyte and trypanosome plasma membranes with time, for Zwittergent 14 (2 and 3mM), Triton X-100 (0.5%(w/v)and n-octyl-β-D-glucopyranoside (50mM) extracts.

The magnitude of the D-glucose transport with time for the erythrocyte derived proteoliposomes is of the same order as quoted by Kasahara and Hinkle (1976). In all cases the erythrocyte derived proteoliposomes demonstrate a rapid initial rate of filling, over the first 10 seconds, slowing as equilibrium is approached. The trypanosome derived proteoliposomes demonstrate an almost linear rate of filling of the proteoliposomes with no initial rapid transport.

For all the detergent derived proteoliposomes the initial rate of filling of the proteoliposomes is greater for erythrocyte derived proteoliposomes, and is linear for the first 10 seconds of incubation. A ten second time point was therefore used to determine the initial rate of D-glucose influx for D-glucose concentrations above 0.1mM.

Table 9 summarises the initial rates of D-glucose transport into the proteoliposomes formed from each

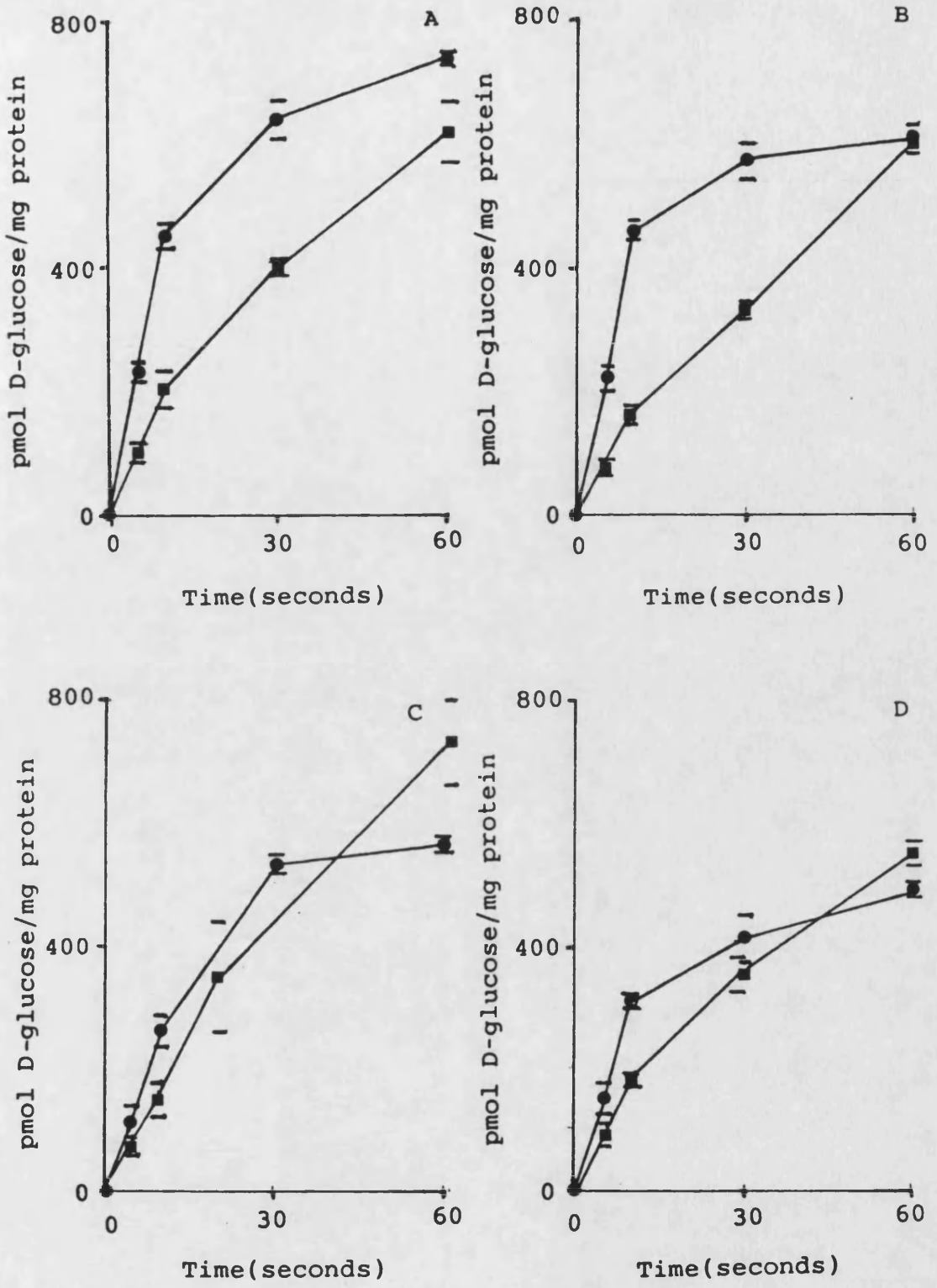


Fig. 30. Zero-trans D-glucose transport into proteoliposomes derived from 0.5%(w/v) Triton X-100 (A), 2mM(B) and 3mM(C) Zwittergent 14 and n-octyl-B-D-glucopyranoside (D) extracts, from human erythrocyte (●) and trypanosome (■) plasma membranes at 0.1mM D-glucose concentration.

Table 9 Summary of initial rates of D-glucose transport (nmol/mg protein/min) for proteoliposomes formed from detergent extracts of trypanosome and erythrocyte plasma membranes by the freeze/thaw/sonication technique.

Detergent	Protein source	D-glucose (mM)						
		.1	.2	.5	1.0	5.0	10.0	20.0
Zwittergent 14 (2mM)	erythrocyte	1.98±.1	4.32±.51	16.2±0	19.64±.71	94.3±1.64	136.7±10.4	-
	trypanosome	.96±.04	-	6.2±.74	-	36±18.0	90±5.8	192±15.7
Zwittergent 14 (3mM)	erythrocyte	1.57±.14	-	-	18.2±2.7	-	67.3±3.2	-
	trypanosome	.9±.17	-	-	10.56±1.59	35.6±6.6	92.3±22.1	188±15.3
TritonX-100 (0.5% (w/v)	erythrocyte	2.67±.11	-	-	26.2±2.62	-	148.9±7.1	-
	trypanosome	1.1±.20	-	-	8.4±3.1	-	97±14.0	218±5.3
n-octyl-B-D-glucopyranoside (50mM)	erythrocyte	2.3±0.9	-	-	15.8±1.9	-	104±17.7	-
	trypanosome	1.2±.33	-	-	9.9±1.4	-	106±9.5	223±26.3

detergent extract of the erythrocyte and trypanosome plasma membranes. The initial rates of D-glucose transport into the proteoliposomes for 2mM Zwittergent 14 extracts, against increasing D-glucose concentration are plotted on Fig. 31.

In all cases the erythrocyte derived proteoliposomes exhibit increasing saturation of transport with increasing D-glucose concentration, suggesting a facilitative transport system is operational. The trypanosome derived proteoliposomes exhibit a linear response to increasing D-glucose concentrations, with no apparent saturation of D-glucose influx, suggesting that no facilitated D-glucose transport system is present.

5.1.2 Reconstitution by the n-octyl-B-D-glucopyranoside detergent dialysis procedure

Treatment of the erythrocyte ghosts with 340mM n-octyl-B-D-glucopyranoside extracted 57% of the erythrocyte plasma membrane protein. Unlike all the other D-glucose transport assays this technique relied upon the decrease in oxygen concentration caused by the activities of glucose oxidase and catalase inside the proteoliposomes. These enzymes yielded D-gluconic acid and $2H_2O$, with the net usage of $1O_2$ (see materials and methods section 10.4.2), in the presence of (transported) D-glucose and dissolved molecular oxygen. From the oxygen consumption, initial rates were calculated.

The initial rates of zero-trans D-glucose transport

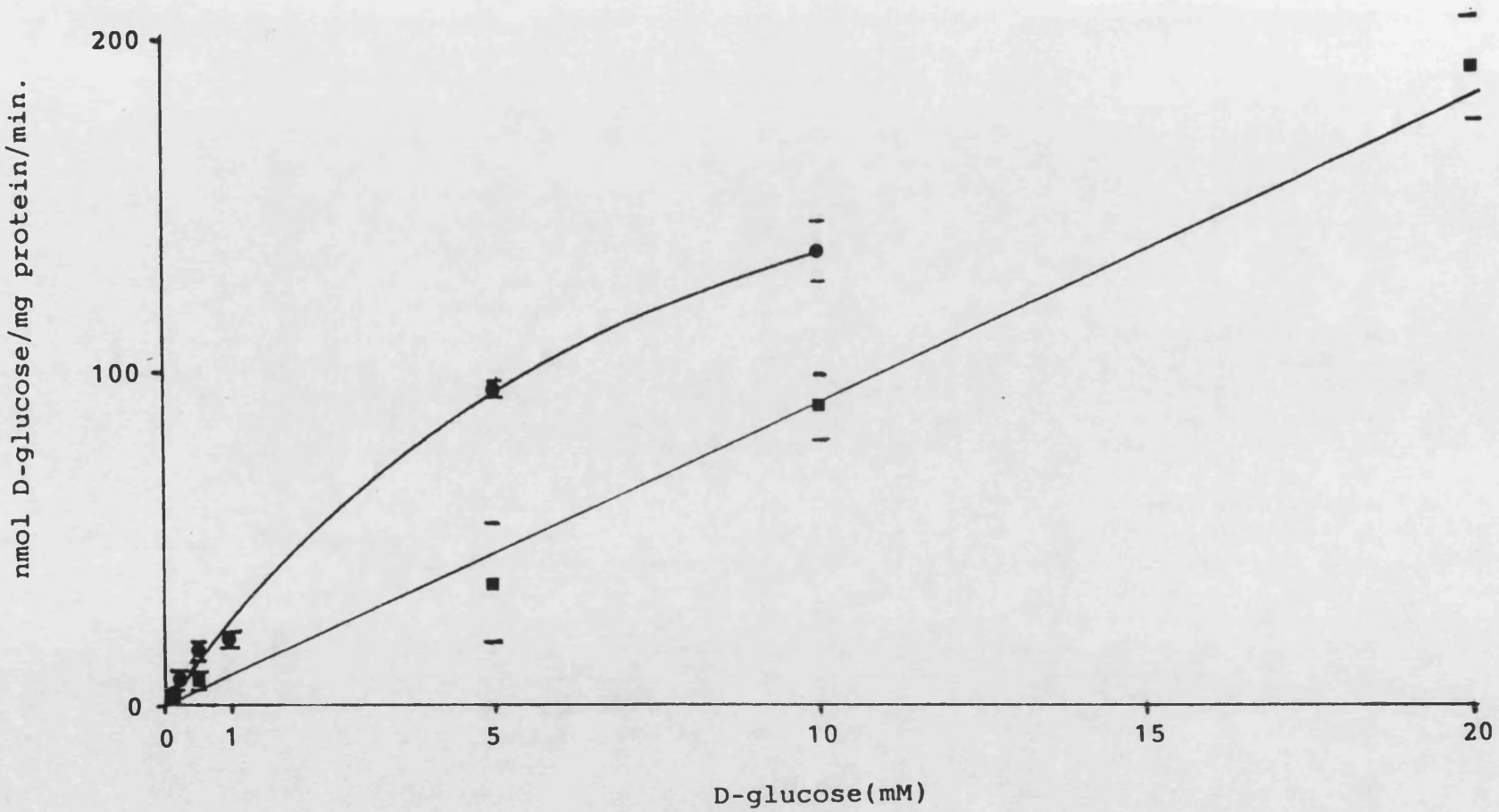


Fig. 31 Zero-trans D-glucose transport, initial rate vs. D-glucose concentration for proteoliposomes produced by the freeze/thaw/sonication technique from 2mM Zwittergent 14 extracts of human erythrocytes (●) and trypanosome (■) plasma membranes.

against D-glucose concentration up to 100mM are shown in figure 32A. Curve 1 represents the D-glucose influx into the proteoliposomes with no inhibitor present. At D-glucose concentrations less than 10mM there is saturation of the influx with increasing concentration. Above 10mM D-glucose the influx is linear with respect to D-glucose concentration. Curve 2 represents the influx of D-glucose into the proteoliposomes in the presence of 1mM p-CPMSA.The linearity of the increase in rate with D-glucose concentration suggests the rate is a "leakage" component, subtraction of this curve from curve 1 gives the final curve, curve 3. This represents the inhibitable components of D-glucose influx ie. that attributable to the D-glucose transporter incorporated into the proteoliposomes. These results are in close agreement with those obtained by Shelton and Langdon (1983) in which 1.6mM phloretin was used in place of 1mM p-CPMSA with the same results. The Km of this system was 14.3mM as determined by a Hanes plot, which compares favourably with the value of 15mM found by Shelton and Langdon (1983).

When erythrocyte "ghosts" were replaced by trypanosome membranes 41% of the total protein was extracted at 340mM n-octyl-B-D-glucopyranoside. Fig. 32B demonstrates the influx of D-glucose into the proteoliposomes against D-glucose concentration. In contrast to the erythrocyte derived proteoliposomes no saturable component could be detected; even at concentrations up to 200mM D-glucose.

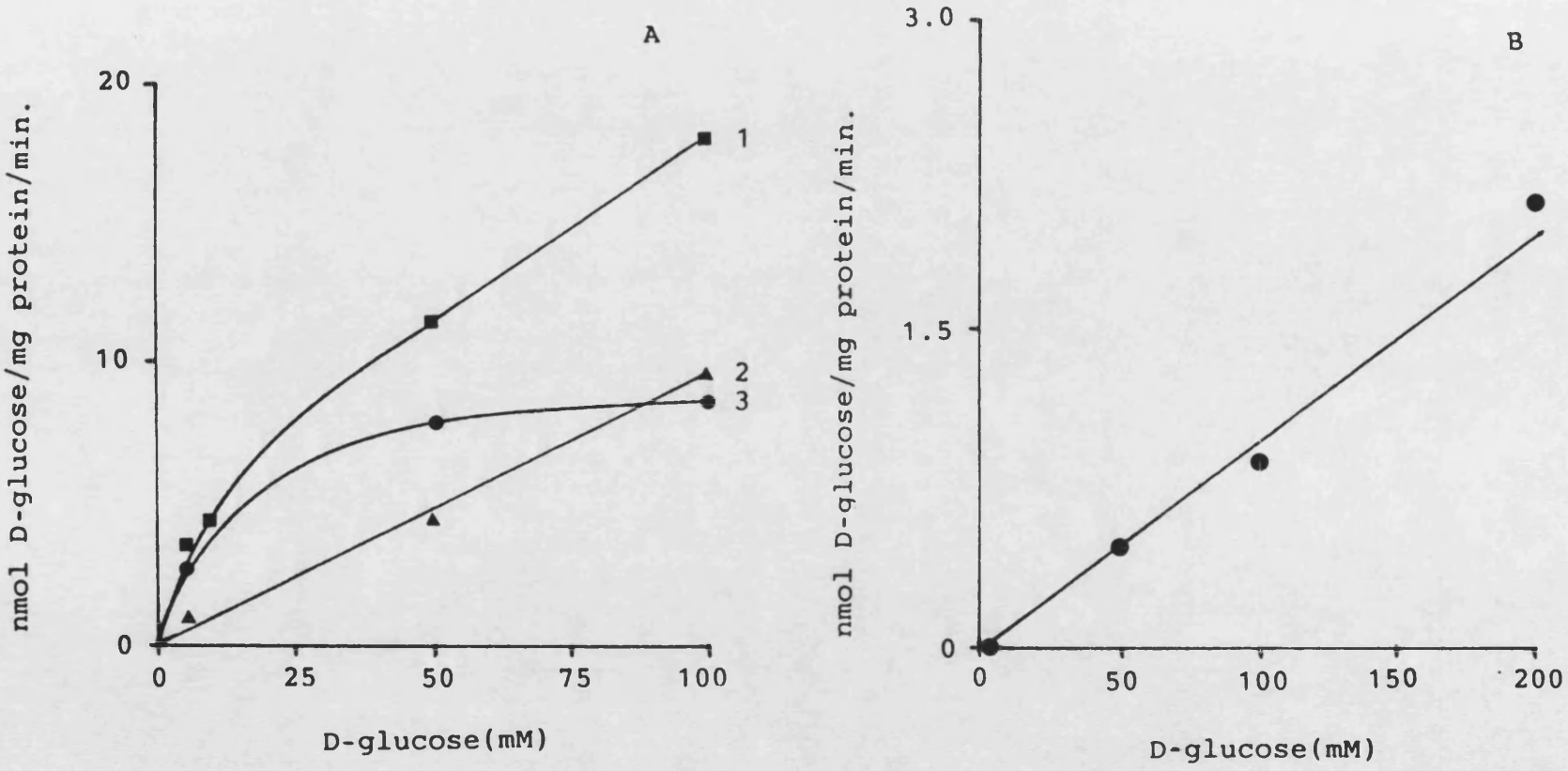


Fig. 32. Zero-trans D-glucose influx into proteoliposomes from n-octyl-B-D-glucopyranoside (340mM) extracts of erythrocyte (A) and trypanosome(B) plasma membranes. Curve 1 is the total D-glucose influx into the proteoliposomes. Curve 2 is the D-glucose influx into the proteoliposomes in the presence of 1mM p-CPMSA and curve 3 is the saturable D-glucose influx achieved by subtraction of curve 2 from curve 1.

For both the erythrocyte and trypanosome plasma membrane derived proteoliposomes in the presence or absenceof p-CPMSA addition of Triton X-100 to give a final concentration of 0.1% (w/v) activated the latent glucose oxidase and catalase activity inside the proteoliposomes.

5.2 INFINITE-TRANS (INFLUX COUNTERFLOW) D-GLUCOSE INFLUX INTO RECONSTITUTED PROTEOLIPOSOMES

The system of measuring D-glucose influx into proteoliposomes was altered to the infinite-trans (influx counterflow) protocol as opposed to the zero-trans protocol because it provided greater sensitivity in detecting true D-glucose transport, rather than non-specific D-glucose influx.

Preloading of the proteoliposomes with 100mM D-glucose competitively inhibited the radiolabelled D-glucose transported into the proteoliposomes from exiting again. This effectively produced a concentration of radiolabelled D-glucose inside the proteoliposomes above that of the equilibrium value, for as long as the intravesicular concentration of unlabelled D-glucose, and the ratio of unlabelled to labelled D-glucose inside the proteoliposomes remained high.

The second advantage of the system was the high internal (trans) D-glucose concentration compared with the external (cis) concentration, minimising the entrance of radiolabelled D-glucose by a leakage component against

the D-glucose concentration gradient.

Finally, the smaller amount of material and time required to undertake infinite-trans assays and the simplicity of the assays made them preferable to the zero-trans technique.

5.2.1 Freeze/thaw/sonication reconstitution of trypanosme plasma membrane proteins

Membrane proteins extracted with Zwittergent 14, lysolecithin and Triton X-100 were reconstituted by the freeze/thaw/sonication technique. Table 10 summarises the influx of D-glucose into proteoliposomes preloaded with 100mM D-glucose, from the detergent extracts at various concentrations. None of the proteoliposomes regardless of detergent or detergent concentration demonstrated influx counterflow facilitated D-glucose transport. A comparison of the influx of D-glucose at 1mM and 100mM for 3mM Zwittergent 14 reconstituted extracts demonstrates a similar linear influx of D-glucose, with no apparent saturation at the higher concentration.

The values for influx into lysolecithin derived proteoliposomes are noticeably lower than those for other detergent extracts (and the lysolecithin 0 value). This was probably caused by the presence of lysolecithin in the proteoliposomes assayed, which weakened the structures and resulted in breakage of the vesicles during washing and filtering. The low critical micelle concentration of lysolecithin made it extremely difficult to remove completely from the detergent extracts.

Table 10 Summary of the infinite-trans D-glucose influx (pmoles) into proteoliposomes prepared by the freeze/thaw sonication technique from detergent extracts of Triton X-100, Zwittergent 14 and lysolecithin. (All values ± S.E. no greater than 23% of the quoted value).

Detergent / Conc. / Time (min)	Triton X-100 (w/v)		Zwittergent (mM)			Lysolecithin (w/v)			
	.25%	.5%	2.0	3.0	3.0*	0	.3%	.6%	1.2%
0.167	14.1	10.9	32	50	5580	3.0	1.2	1.4	1.4
2.0	20.3	24.4	69	95	11250	9.1	1.9	1.9	2.8
10.0	39.8	40.2	173	140	19620	18.6	3.1	3.8	14.0
30.0	90.0	88.0	294	257	25650	32.3	6.7	10.3	17.0
90.0	171.0	157.0	381	311	40500	85.0	11.3	13.5	27.5
150.0	-	-	526	432	56250	-	-	-	-

* Denotes 100mM extravesicular D-glucose concentration.

Figs. 33, 34 and 35 show the percentage of the total protein extracted by each detergent, the total dihydrolipoamide dehydrogenase activity retained in the extracts and the post extraction pellets, the percentage of the total protein reconstituted into the proteoliposomes, and the total dihydrolipoamide dehydrogenase activity reconstituted into the proteoliposomes.

Triton X-100 extracts demonstrate an increasing release of protein and dihydrolipoamide dehydrogenase with detergent concentration, reaching a peak at 0.5% (w/v) Triton X-100. A maximum of 10% of the total protein was reconstituted into the proteoliposomes (0.5% (w/v) Triton X-100 extract), retaining 15% of the total dihydrolipoamide dehydrogenase activity.

Both Zwittergent 14 and lysolecithin released greater amounts of protein from the membranes that Triton X-100. Concentrations of lysolecithin above 0.3% (w/v) did not release significantly greater amounts of protein, and total dihydrolipoamide dehydrogenase activity decreased in the supernatants. Total protein reconstituted was a maximum of 4% of the total (0.6% (w/v) extract) with the reconstituted dihydrolipoamide dehydrogenase activity not exceeding 2.5% of the total, both figures significantly lower than achieved with Triton X-100.

Zwittergent 14 extraction released a maximum of 50.6% of the total protein at 10mM, which was not significantly greater than that achieved at 3mM detergent (45%). The 3mM Zwittergent 14 extract reconstitution incorporated 6.1% of the total protein and 8.3% of the

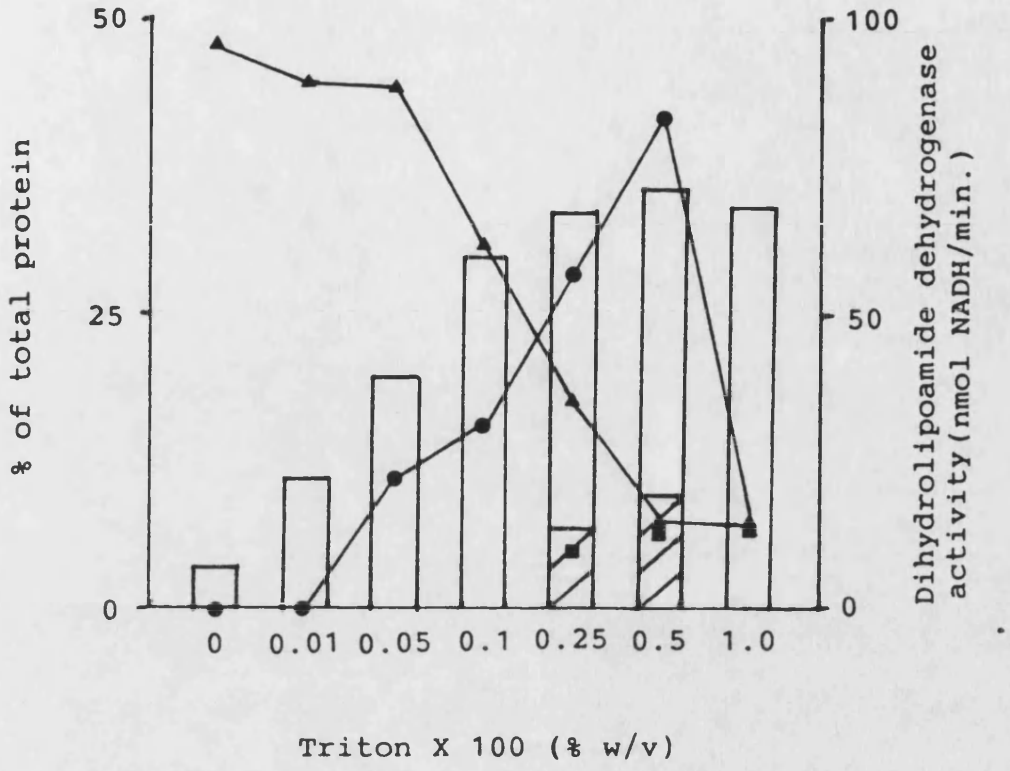


Fig. 33. Triton X-100 extraction of trypanosome plasma membranes showing % total protein in extract (□), % of the total protein reconstituted by the freeze/thaw/sonication method into proteoliposomes (▨), total DHLIP DH activity in extracts (●), post extract pellets (▲), and reconstituted proteoliposomes (■).

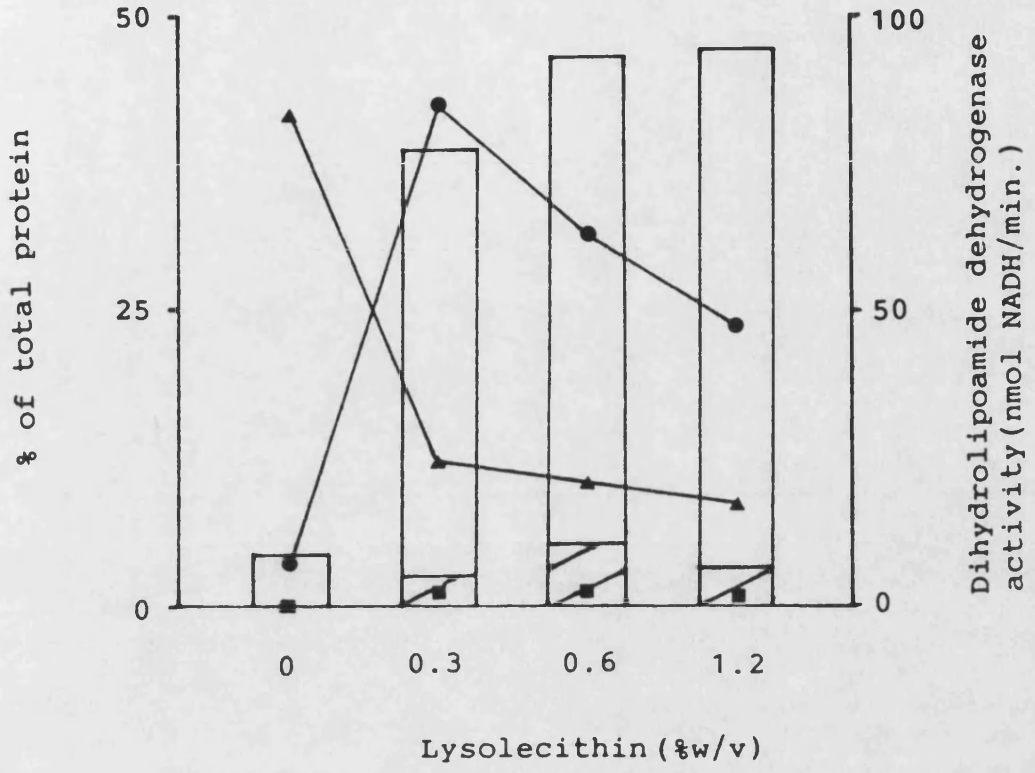


Fig. 34. Lysolecithin extraction of trypanosome plasma membranes showing % total protein in extract (□), % of the total protein reconstituted by the freeze/thaw/sonication method into proteoliposomes (▨), total DHLIP DH activity in extracts (●), post extract pellets (▲), and reconstituted proteoliposomes (■).

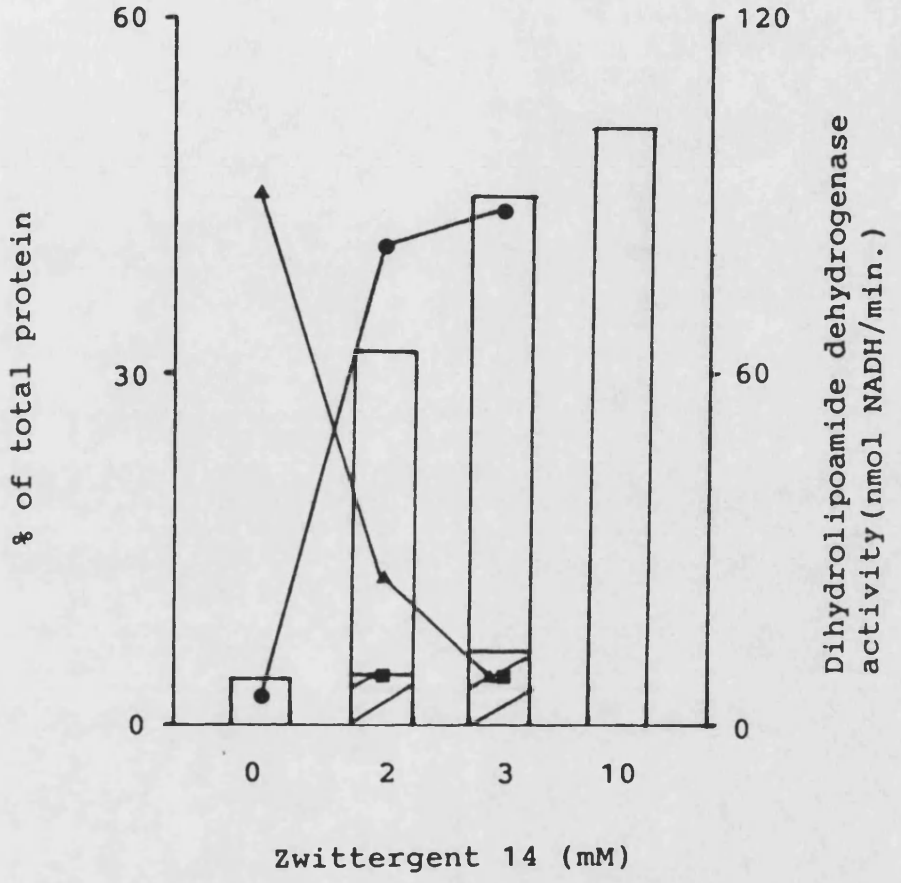


Fig. 35. Zwittergent 14 extracted trypanosome plasma membranes showing % total protein in extract (□), % total protein reconstituted by the freeze/thaw/sonication method into proteoliposomes (▨), total DHLIP DH activity in extracts (●), post extract pellets (▲), and reconstituted proteoliposomes (■).

total dihydrolipoamide dehydrogenase activity.

5.2.2 Detergent dilution reconstitution procedure

N-octyl-B-D-glucopyranoside (1.5% (w/v)) extracted 47% of the total erythrocyte "ghost" protein. Four and a half percent of the protein was reconstituted into the proteoliposomes, which exhibited infinite-trans D-glucose facilitated transport, fig. 36. Substitution of trypanosome plasma membranes in place of erythrocyte "ghosts" released 31 to 38% of the total membrane protein. Total protein incorporated into the proteoliposomes ranged from 4.1 to 7.6% of the total protein. Fig. 37 shows the infinite-trans D-glucose influx and 1mM sucrose influx into proteoliposomes formed from the trypanosome lipid, α-lecithin and egg phosphatidyl choline preloaded with 100mM D-glucose. The influx of D-glucose into the trypanosome and egg phosphatidyl choline lipid proteoliposomes is greater than those with L-α-lecithin lipid; however, none show rapid accumulation of D-glucose above the equilibrium value expected of facilitated transport.

The higher values attained by trypanosome lipid and L-α-lecithin may be a result of the larger size and lower homogeneity of the proteoliposome population, caused by the inability to sonicate the lipid to clarity before use.

The detergent dilution procedure was applied to the 2 other high critical micelle concentration detergents, decanoyl-N-methyl-glucamid (MEGA 10) and

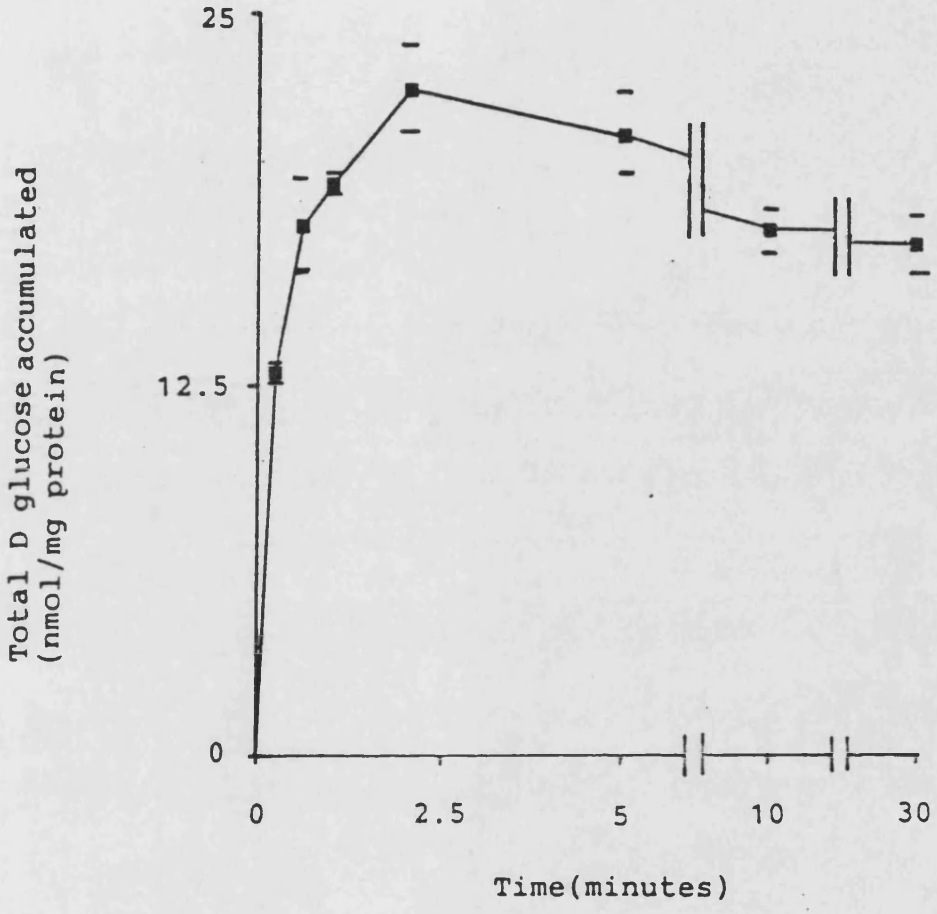


Fig. 36. Infinite-trans D-glucose transport into proteoliposomes formed by detergent dilution from 1.5% (w/v) n-octyl-B-D-glucopyranoside extract of erythrocyte "ghosts".

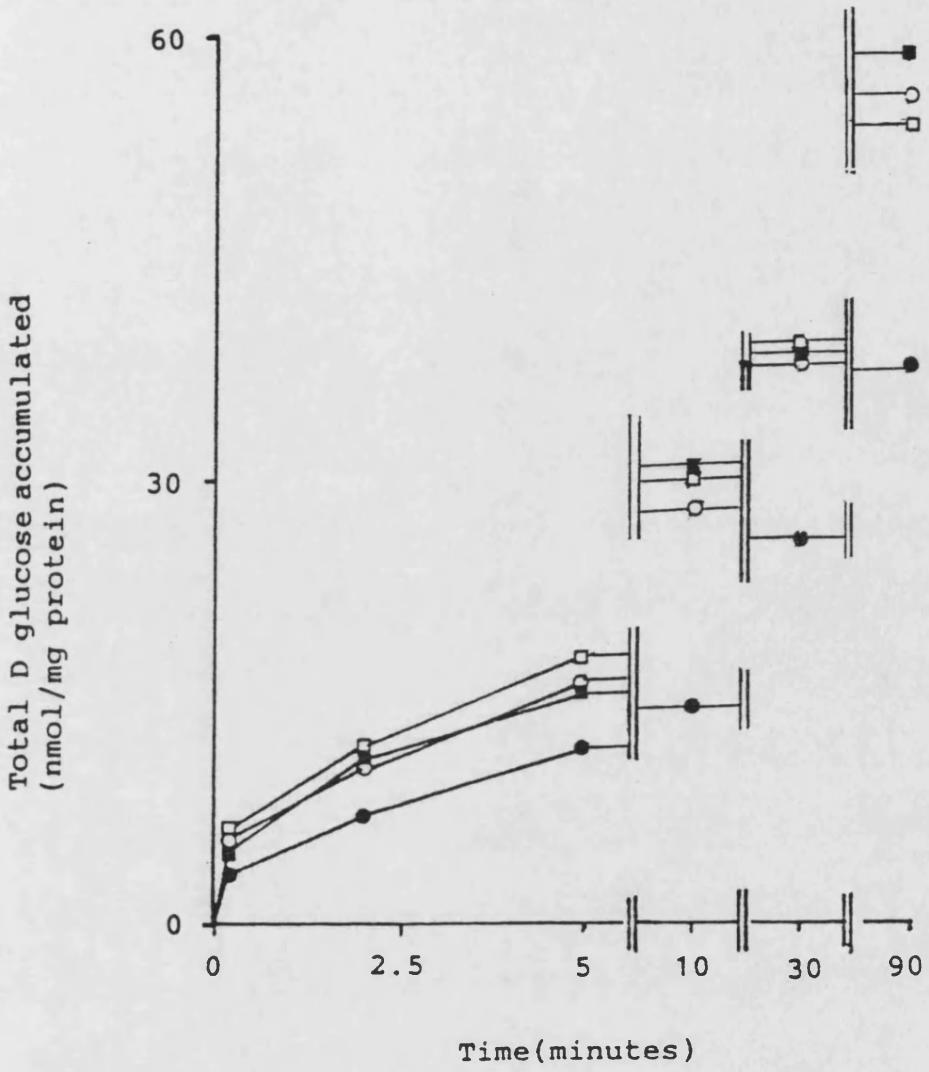


Fig. 37. Infinite-trans D-glucose influx into proteoliposomes formed by the detergent dilution procedure in the presence of L-α-lecithin(●), egg yolk phosphatidyl choline(○), trypanosome lipid(□), and 1mM sucrose influx into trypanosome lipid detergent extract proteoliposomes (■). All values ± SE of no greater than 13% of the given value.

sodium cholate. Fig. 38 and 39 give the percentage of the total protein extracted at each detergent concentration, the total dihydrolipoamide dehydrogenase activity in the extract and residual pellet, the % of the total protein reconstituted, and the total dihydrolipoamide dehydrogenase reconstituted into the proteoliposomes.

Sodium cholate extracted a maximum of 43.4% of the total protein at 2.0% (w/v) detergent, however the release of protein and dihydrolipoamide dehydrogenase did not increase significantly above 1.0% (w/v) sodium cholate, fig. 38. Reconstitution of the 2.0% (w/v) extract incorporated 7.2% of the total protein and 11.3% of the total dihydrolipoamide dehydrogenase activity.

No sodium cholate reconstituted extract demonstrated the accumulation of D-glucose to a figure above the equilibrium value, followed by a decrease with time, Table 11. Decanoyl-N-methyl-glucamid extracted a maximum of 37.5% of the total protein at 1.0 (w/v) detergent. Above 0.25% (w/v) detergent however, total dihydrolipoamide dehydrogenase activity in both the extract and the residual post-extraction pellet decreased. Maximum protein was reconstituted into the proteoliposomes from the 0.5% (w/v) extract (8.4% of the total) and 5.8% of the total dihydrolipoamide dehydrogenase activity. Infinite-trans D-glucose transport could not be detected in the proteoliposomes from any of the detergent extracts, and exhibited similar D-glucose influxes to those of sodium cholate, Table 11.

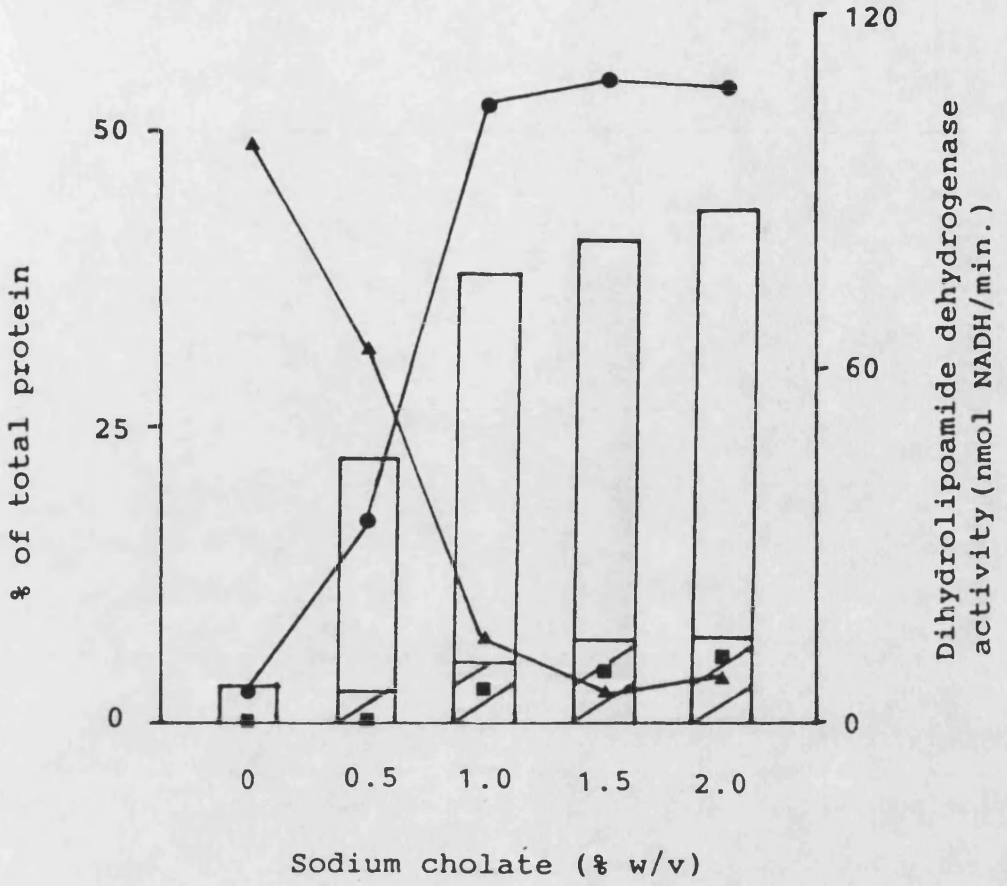


Fig. 38. Sodium cholate extraction of trypanosome plasma membranes showing % of the total protein in the extracts (□), % of the total protein reconstituted by the freeze/thaw/sonication method into proteoliposomes (□), total DHLIP DH activity in extracts (●), post extract pellets (▲), and reconstituted proteoliposomes (■).

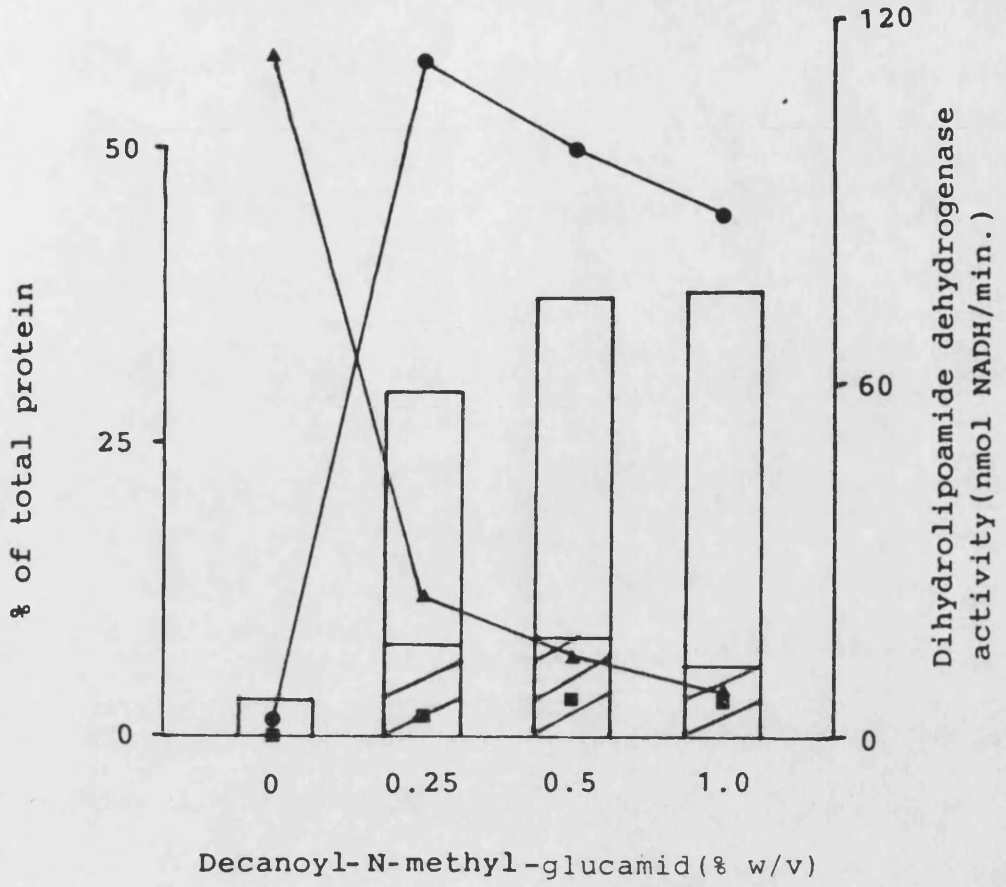


Fig. 39. Decanoyl-N-methyl-glucamid (MEGA 10) extraction of trypanosome plasma membranes showing % total protein in the extracts (□), % of the total protein reconstituted by the freeze/thaw/sonication method into proteoliposomes (▨), total DHLIP DH activity in extracts (●), post extract pellets (▲), and reconstituted proteoliposomes (■).

Table 11 Summary of the infinite-trans D-glucose influx(pmoles) into proteoliposomes formed by the freeze/thaw/sonication procedure applied to sodium cholate and decanoyl-N-methyl-glucamid extracts of trypanosome plasma membranes (all values ± SE no greater than 16% of the quoted value).

Detergent / Conc. / Time (min)	Sodium cholate (w/v)					Decanoyl-N-methyl-glucamid (w/v)			
	0	.5%	1.0%	1.5%	2.0%	0	.25%	.5%	1.0%
0.167	58.3	32.3	24.6	30.2	27.3	6	13.0	5.4	26.6
2.0	95.0	65.0	29.0	35	48.1	9.3	14.1	12.2	44.8
10.0	127	95.6	49.0	50.3	69.7	18.8	23.0	13.2	73.3
30.0	142	142	57.0	74.5	101.2	21.1	34.5	19.6	86.4
90.0	199	163	76.0	83.5	121.5	36.2	55.0	34.3	156

5.2.3 Direct incorporation of plasma membrane vesicles into L-α-lecithin vesicles

The proteoliposome pellet collected after the freezing and thawing of plasma membrane vesicles and L-α-lecithin vesicles retained 54% of the total initial protein. Fig. 40 shows the infinite-trans D-glucose influx into the proteoliposomes with time. Accumulation of D-glucose did not demonstrate facilitated transport characteristics, but rather a non-specific influx component.

Proteoliposomes formed by incubation with lysolecithin retained 25% of the total initial protein and 17% of the total initial dihydrolipoamide dehydrogenase activity. Infinite-trans D-glucose transport exhibited a similar influx profile as those formed by the freeze/thaw/sonication process, but did not demonstrate a facilitated D-glucose transport process, fig. 41.

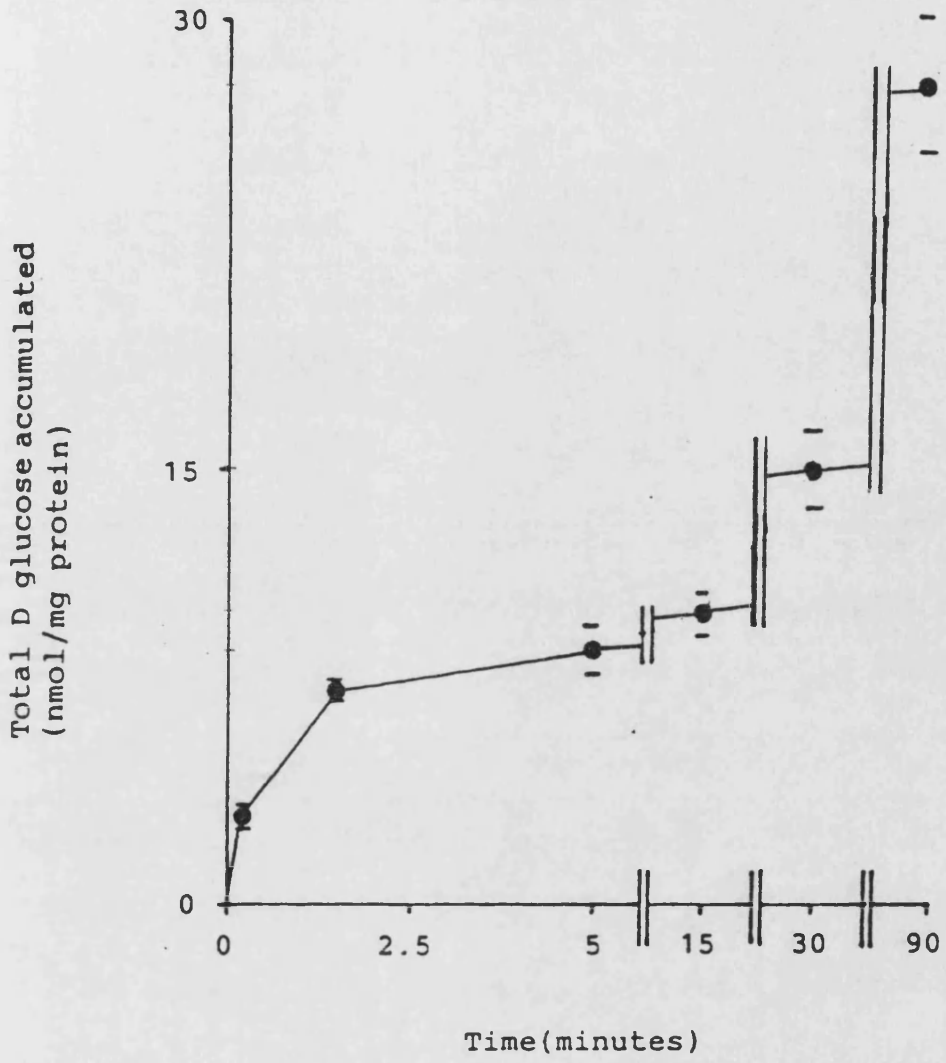


Fig. 40. Infinite-trans D-glucose influx into proteoliposomes formed by direct incorporation of plasma membrane vesicles into L-α-lecithin vesicles by the freeze/thaw/sonication technique.

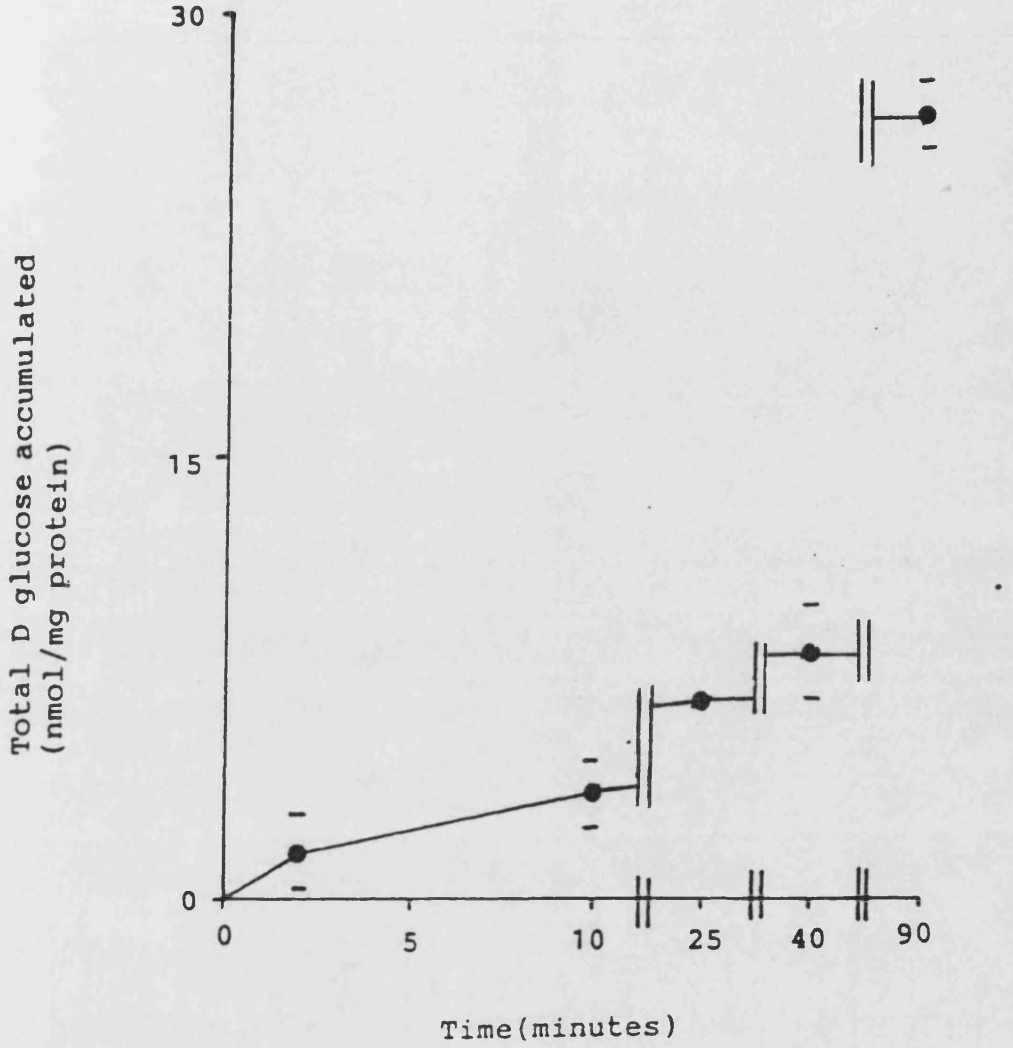


Fig. 41. Infinite-trans D-glucose influx into proteoliposomes formed by the direct incorporation of plasma membrane vesicles into L-α-lecithin vesicles in the presence of lysolecithin.

6.0 SUGAR TRANSPORT IN TRYPANOSOME PLASMA MEMBRANE VESICLES

Initial D-glucose transport studies were performed, using the zero-trans protocol, ie. no preloading of the vesicles with unlabelled sugar. Under this regimen the total counts found in the plasma membrane vesicles was very low. This made determination of influx rates very problematical since even small aberrations produced significant fluctuations in values, and determination of the ideal time for measurement of initial rates and reproducibility of results was extremely difficult. Transferral to the infinite-trans (influx counterflow) protocol with trans D-glucose concentration 100mM, and cis concentration 1mM overcame the problems associated with the zero-trans protocol.

Figure 42 demonstrates the time course for accumulation of radiolabelled D-glucose in the plasma membrane vesicles preloaded with 100mM D-glucose, with extravesicular concentration of 1mM D-glucose. Also shown are the time courses for vesicles containing 100mM D-glucose but with,

(a) 100mM D-glucose extravesicular concentration, and

(b) 1mM extravesicular D-glucose and 100mM mannitol in all buffers (including stopping buffer).

At 1mM D-glucose extravesicular concentration the vesicles demonstrate an accumulation of radiolabelled D-glucose to a concentration above the equilibrium value, subsequently decreasing to the equilibrium value with increasing time. The 100mM D-glucose time-course produces

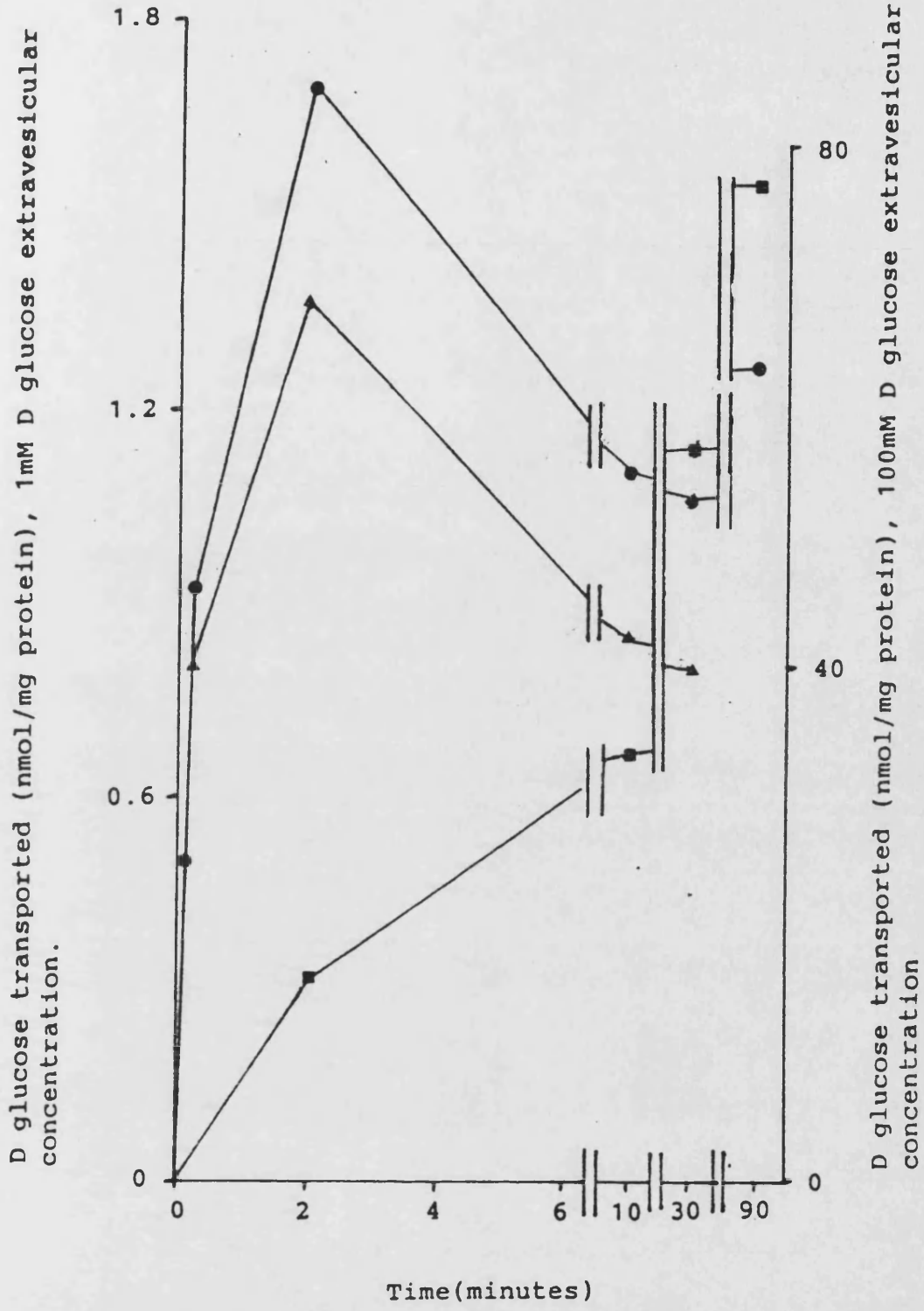


Fig. 42. Time course for infinite-trans counterflow D-glucose transport into plasma membrane vesicles at 1mM D-glucose (●—●), 1mM D-glucose, 100mM mannitol (▲—▲), and 100mM D-glucose (■—■) extravesicular concentration. All values ± SE of 13% or less.

a gradual influx of radiolabelled D-glucose with time and an initial 10 second value less than 100 times the value for 10 seconds at 1mM D-glucose, suggesting saturation of the influx process.

The final plot of 1mM D-glucose influx with 100mM mannitol present, is similar in size and shape to that obtained without the mannitol present. This indicates there is not a major osmotic effect inducing D-glucose influx.

The influx of radiolabelled 1mM D-glucose was linear for the first 10 seconds of the time course, and so this time point was chosen to calculate the initial rates of D-glucose transport with increasing extravesicular D-glucose concentrations of 1 to 100mM.

The internal volume available for D-glucose accumulation was calculated from the total cpm of the sample at equilibrium after 90 minutes incubation, and the total radiolabel present in the total assay volume of 200µl. These gave an internal volume of 0.029±0.0025µl per 2µl of plasma vesicles, equivalent to 5.07±0.84µl per milligram of protein present.

An attempt to increase the entrapment of plasma membrane vesicles in the 0.22µm millipore filters by pre-soaking with poly-L-lysine (as suggested by Franzusoff and Cirillo (1983a) on the plasma membrane vesicles produced from yeast) did not improve the retention; using the criterion of increased counts trapped on the filters as the definitive measurements rather than trapped protein.

The infinite-trans sugar transport profiles for 1-deoxy-D-glucose (1DOG) and 6-deoxy-D-glucose (6DOG) are shown in fig. 43 A and B. These exhibit the same time course characteristics as D-glucose suggesting a facilitated transport process.

6.1 DETERMINATION OF THE MICHAELIS CONSTANT (Km) AND Vmax FOR SUGAR TRANSPORT IN PLASMA MEMBRANE VESICLES

The Km and Vmax for sugar transport using the infinite-trans protocol were calculated from the Hanes-Woolf plot of [S]/V vs. [S]. The best fit of the data was determined using the least squares method, computed according to the program devised by Cleland (1967). This program fitted the raw data to 3 rate equations to determine,

1) The kinetic constants with no treatment of the data,
2) If the raw data included a leakage or non-saturable component, which could then be allowed for in the raw data, and the kinetic constants calculated from the residual saturable transport component,
3) If the raw data included 2 saturable transport components, for which the kinetic constants of both could be calculated.

Figs. 44, 45 and 46 represent the Hanes-Woolf plots for D-glucose, 1-deoxy-D-glucose and 6-deoxy-D-glucose as fitted to the data by the computer program of Cleland (1967). In all three cases no "leakage" (non-specific transport) or dual transport processes could be detected. The best fit of the data was found

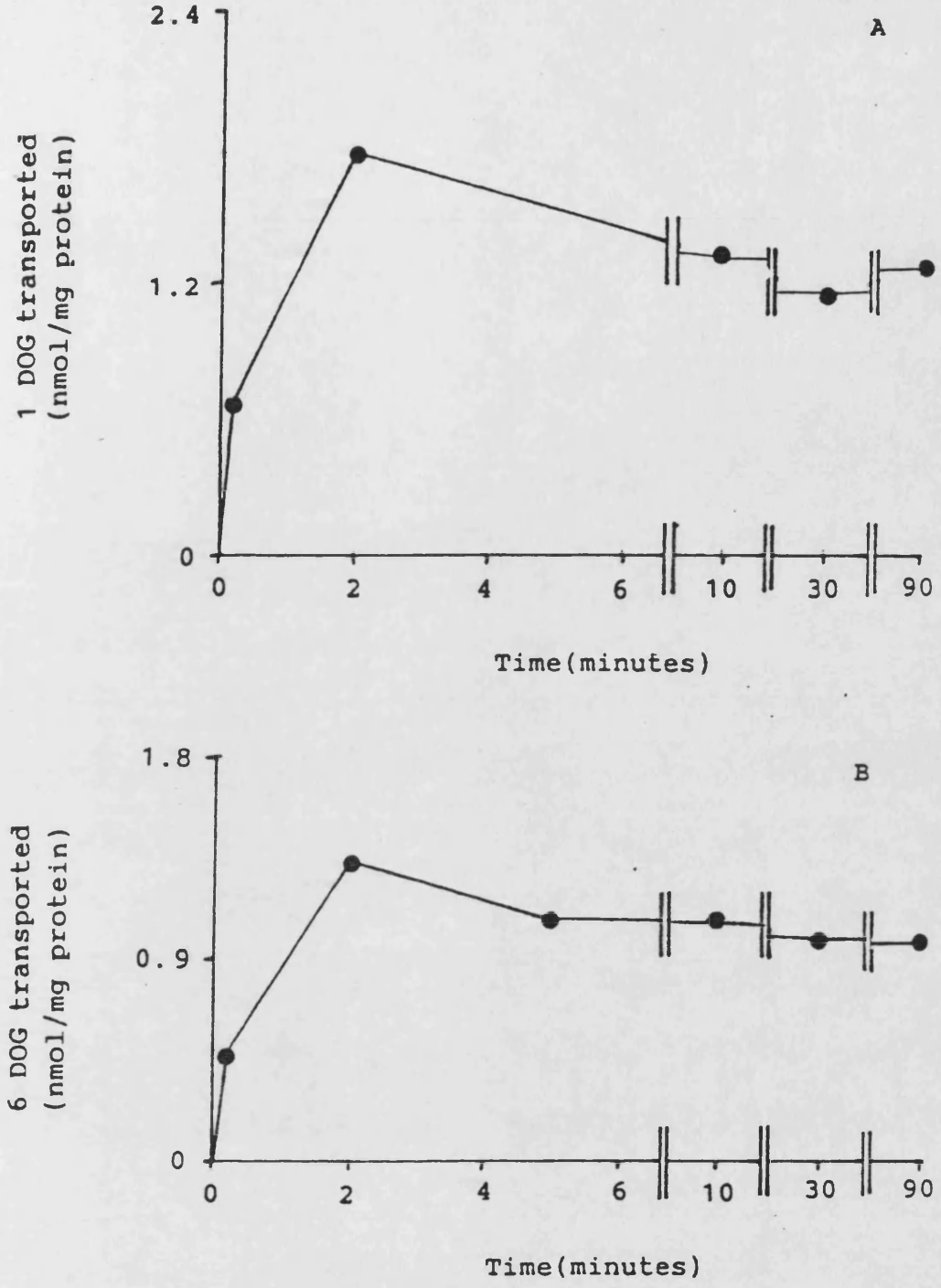


Fig. 43. Infinite-trans (influx counterflow) sugar transport into plasma membrane vesicles with time for 1mM 1DOG (A) and 1mM 6DOG (B). All values ± SE of 18% or less.

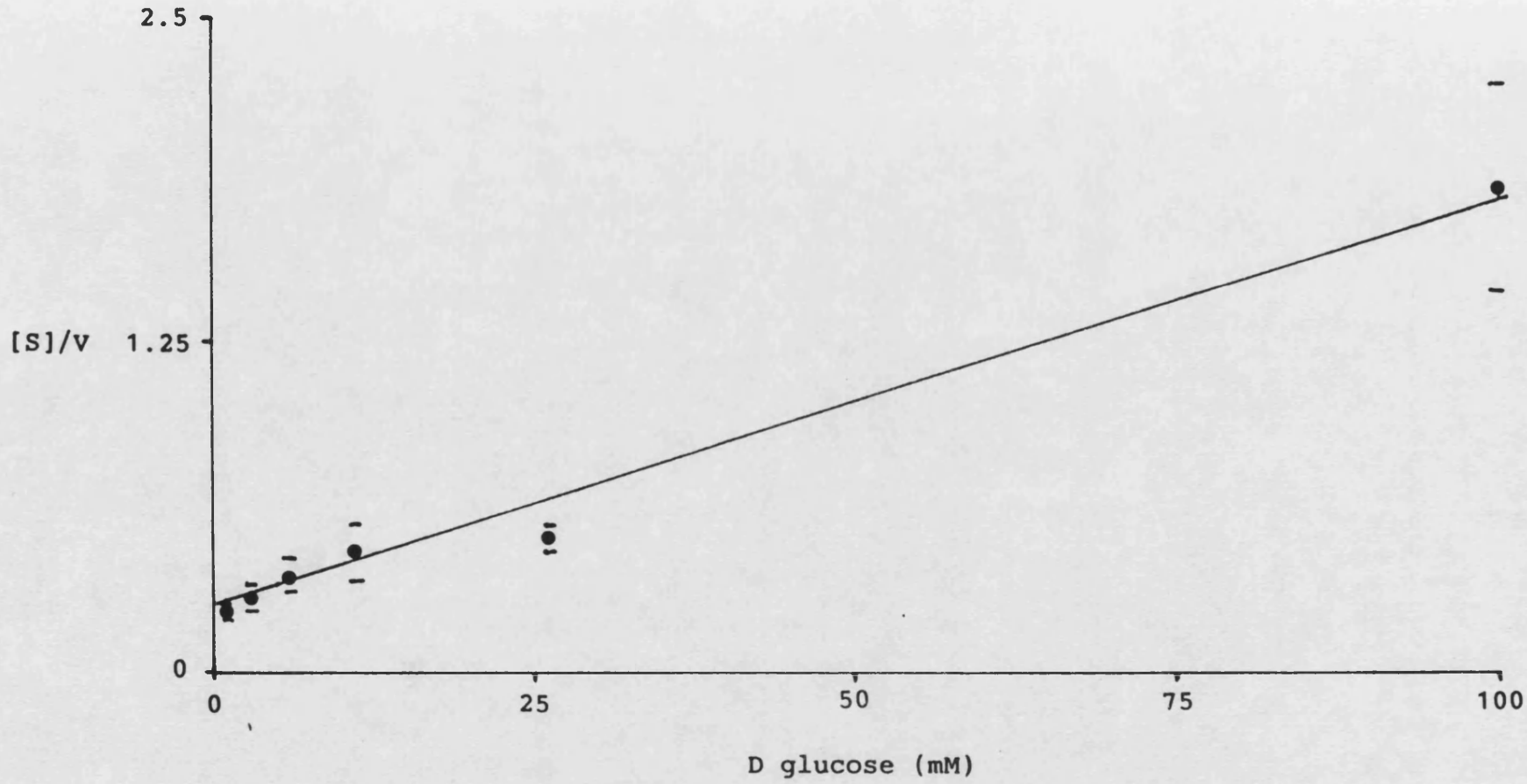


Fig. 44. Hanes plot for D-glucose to determine the Km and Vmax for D-glucose transport into plasma membrane vesicles preloaded with 100mM D-glucose.

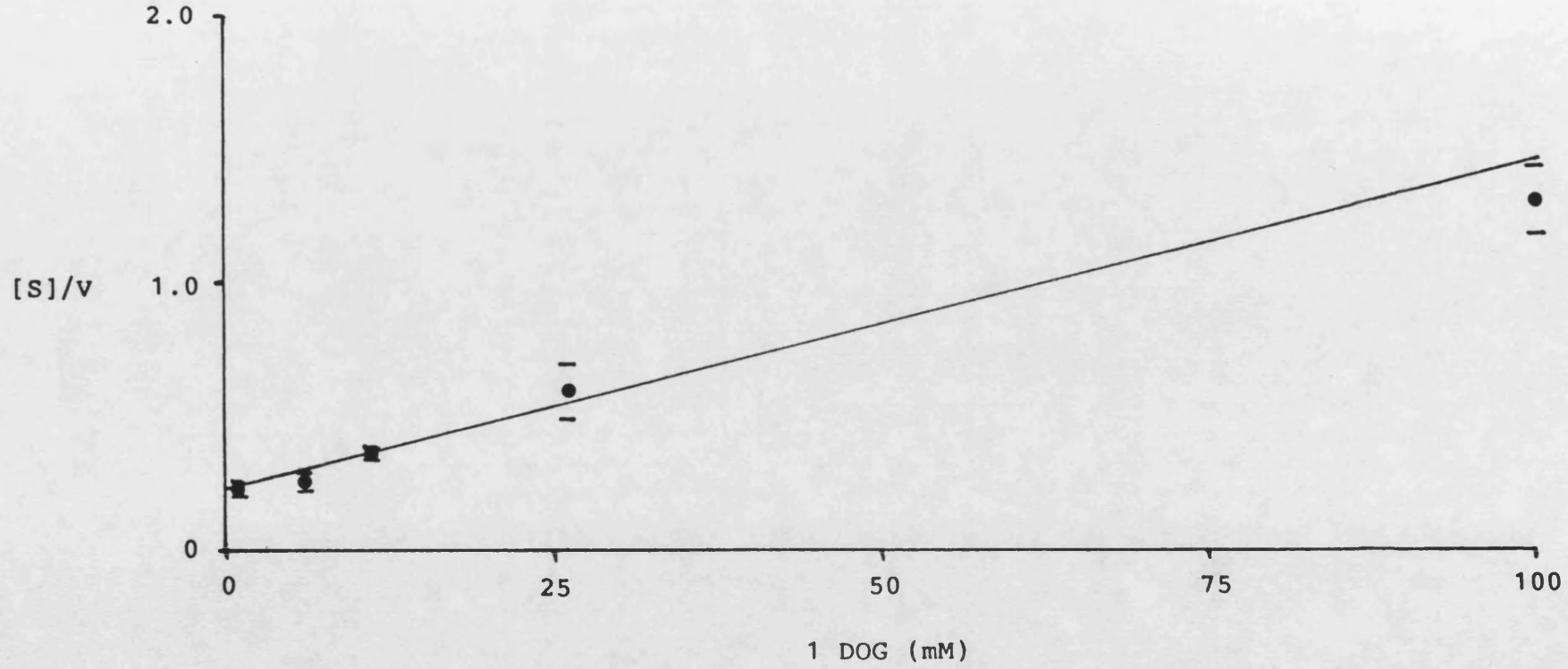


Fig. 45 Hanes plot for 1-deoxy-D-glucose (1DOG) to determine the Km and Vmax for 1DOG transport into plasma membrane vesicles preloaded with 100mM 6DOG.

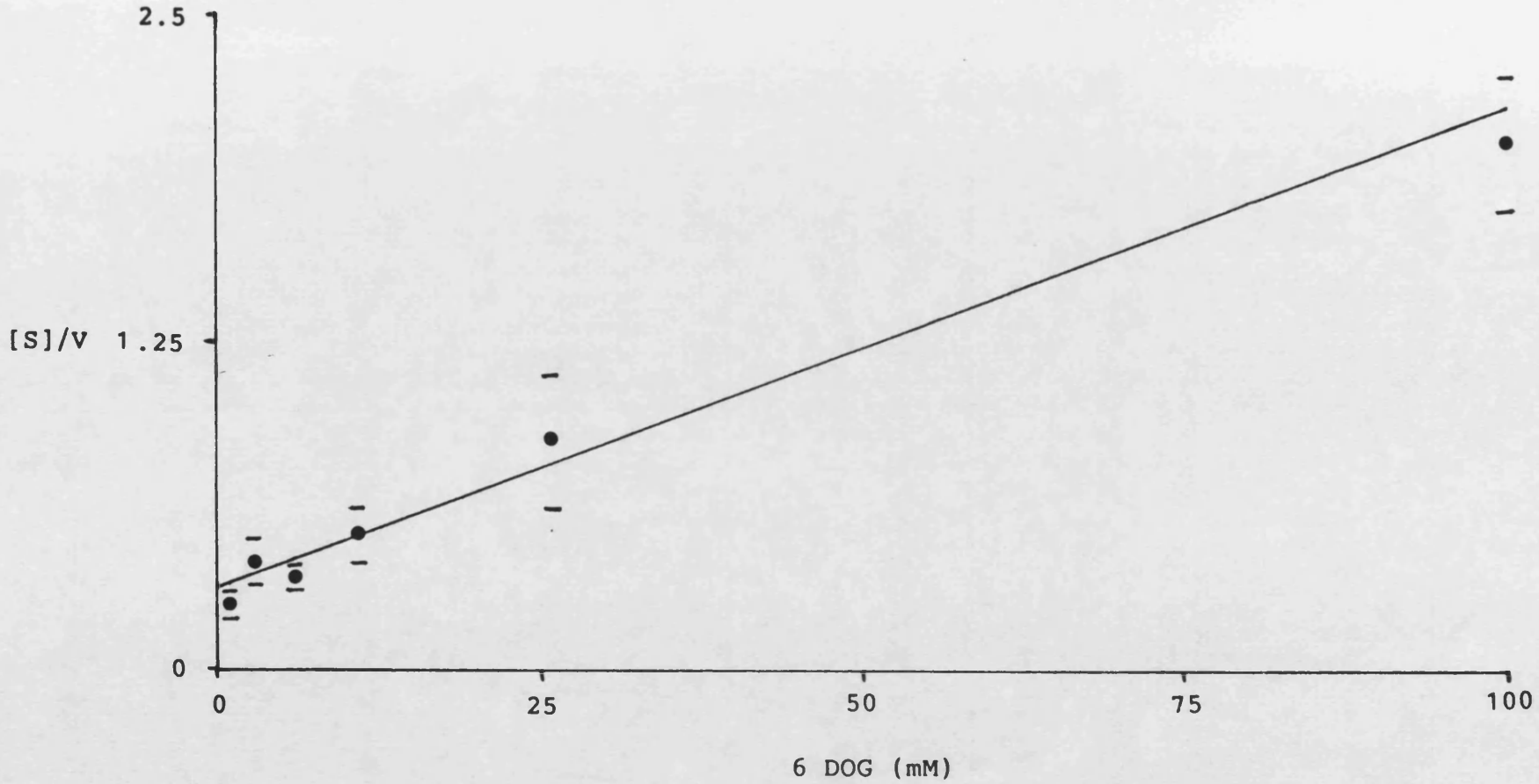


Fig. 46. Hanes plot for 6-deoxy-D-glucose (6DOG) to determine the Km and Vmax for 6DOG transport into plasma membrane vesicles preloaded with 100mM 6DOG.

using the untreated values.

The Km and Vmax of D-glucose, 1DOG and 6DOG are shown in the table below.

Sugar	Michaelis constant (Km) (mM)	Maximum velocity(Vmax) nmol sugar/mg prot/min
D-glucose	15.66±2.8	62.9±7.54
1DOG	17.26±2.4	80.28±7.0
6DOG	16.16±3.8	53.67±8.26

6.2 INHIBITION OF INFINITE-TRANS SUGAR TRANSPORT IN PLASMA MEMBRANE VESICLES BY CARBOHYDRATES

Table 12 summarises the percentage inhibition of infinite-trans sugar transport into plasma membrane vesicles preloaded with 100mM sugar (either D-glucose, 1DOG or 6DOG) with extravesicular sugar concentration 1mM and inhibitor concentration 25mM unless otherwise stated.Phloridzin was used at 2.2mM (1g/l) because of its insolubility above that figure.

All values are the % inhibition after 2 minutes of incubation, as these represent the maximum accumulation of sugar substrate inside the vesicles.

The patterns of inhibition of D-glucose, 1DOG and 6DOG were consistent for most of the inhibitors

Table 12 Summary of the % inhibition of 1mM sugar (D-glucose, 1DOG and 6DOG) infinite-trans transport, by inhibitor (concentration in mM in brackets). All values are ± standard deviation.

Substrate (1mM) / Inhibitor (mM)	D-glucose	1DOG	6DOG
Substrate(100)	89.7±.98	77±1.4	69.9±0.35
D-glucose(25)	58.3±6.8	41.5±3.2	58.4±2.5
D-galactose(25)	51±1.3	-	33±23.3
D-mannose(25)	34.3±3.0	35±2.1	32±1.8
D-glucosamine (25)	59.5±4.6	38±2.1	41.8±2.6
1DOG(25)	49.0±4.9	40±4.2	52.2±4.7
6DOG(25)	48.0±3.5	34±11	47.6±7.6
Maltose(25)	16.5±8.8	33.3±5.5	18.0±0.7
5-thio-D-glucose(25)	50.5±11.7	47.5±6	41.1±4.0
N-acetyl-D-glucosamine(25)	26.6±4.1	38.5±7.4	42.7±3.4
Mannitol(25)	0	0	0
3-O-methyl-D-glucose(25)	43±2.8	37±2.1	51.2±1.1
D-fructose(25)	25.5±.13	-	-
Glycerol(25)	0	-	-
Phloridzin(2.2)	15.9±3.0	23±2.1	0

A - denotes no observation.

tested. In all cases 100mM sugar gave the greatest inhibition of filling ranging from 69.9% for 6-deoxy-D-glucose to 89.7% for D-glucose. Neither mannitol nor glycerol produced any inhibition. D-fructose, maltose and phloridzin induced moderate inhibition of transport of 33.5% or less for D-glucose, 1DOG and 6DOG.

Inhibition of 1DOG varied from that of 6DOG and D-glucose in that it was inhibited less by D-glucose, 1DOG, 6DOG, and D-glucosamine than were D-glucose and 6DOG, and was inhibited to a greater extent by maltose.

D-glucose, D-galactose, D-glucosamine, 5-thio-D-glucose, 1DOG, 6DOG, D-mannose, N-acetyl-D-glucosamine and 3-O-methyl-D-glucose all gave greater than 26.6% inhibition. D-mannose (with the exception of N-acetyl-D-glucosamine inhibition of D-glucose transport) gave a lower inhibition of D-glucose, 1DOG and 6DOG transport than N-acetyl-D-glucosamine or D-glucosamine.

6.3 INHIBITION OF INFINITE-TRANS D-GLUCOSE TRANSPORT IN PLASMA MEMBRANE VESICLES BY CHEMICAL MODIFYING AGENTS

D-glucose transport was inhibited by 2,4,dinitro-fluorobenzene, 2-hydroxy-5-nitrobenzyl bromide, and by N-ethylmaleimide in the plasma membrane vesicles without preincubation with inhibitor. The inhibitor was added to the vesicles with the diluting buffer. Inhibition by mercuric chloride could not be measured because it had the effect of "clumping" the vesicles

together at concentrations as low as 0.5mM, producing erratic and nonsensical results.

Fig. 47 shows graphically the % inhibition of D-glucose transport by all these inhibitors at the 2 minute incubation time point. N-ethylmaleimide and 2,4 dinitrofluorobenzene inhibited transport by up to 34% with inhibition increasing with increasing inhibitor concentration. In contrast, 2-hydroxy-5-nitrobenzyl bromide demonstrated greater inhibition which began to plateau after 2mM inhibitor, reaching a maximum value of 69.5% at 5mM inhibitor.

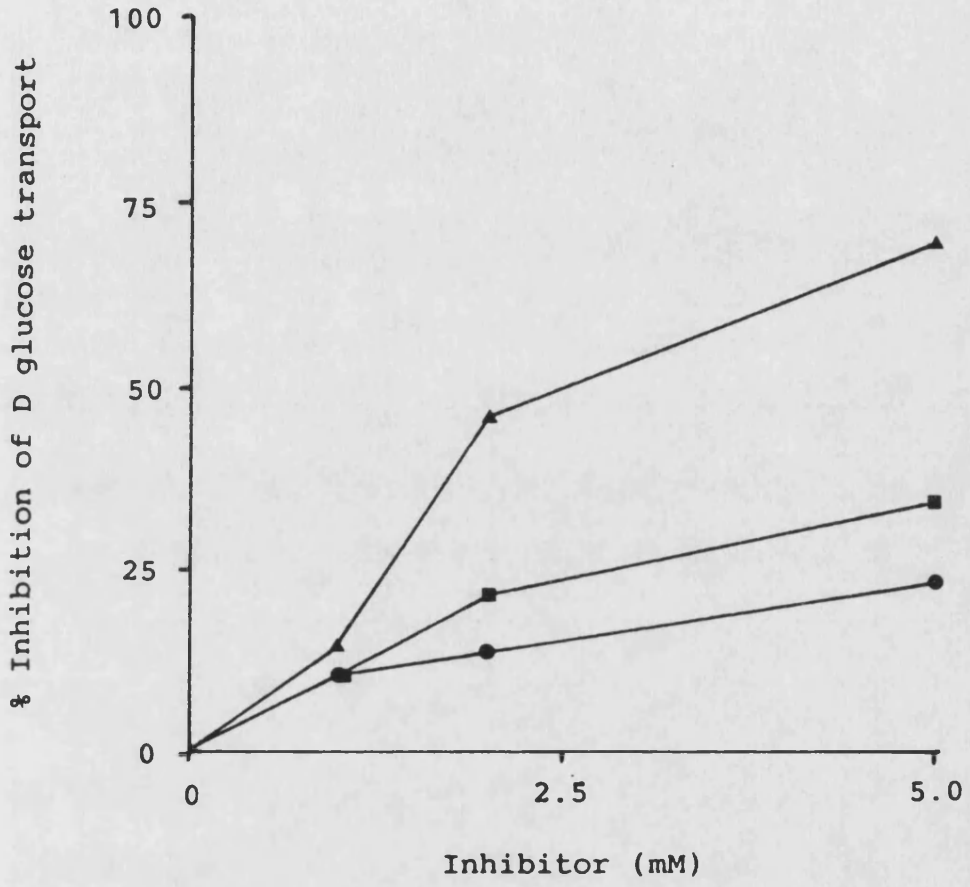


Fig. 47. % inhibition of infinite-trans D-glucose (1mM) transport at increasing inhibitor concentrations for 2,4 dinitrofluorobenzene (■), 2-hydroxy-5-nitrobenzyl bromide (▲), and N-ethylmaleimide (●). All values ± SE no greater than 19% of the given value.

7.0 INHIBITION OF 6DOG TRANSPORT BY CHEMICAL MODIFYING AGENTS IN INTACT VIABLE TRYPANOSOMES

Viable trypanosomes preincubated with 2-hydroxy-5-nitrobenzyl bromide at 3mM final concentration transported 6DOG (zero-trans) into themselves at a rate less than 8% of that achieved in the absence of inhibitor, fig. 48. Preincubation for as little as 1 minute almost totally inhibited influx; however under microscopic investigation no lysis of the trypanosomes could be observed, and 90% retained their motility. The motility decreased until 10 minutes of preincubation at which time no motility could be observed. These cells still appeared intact as determined by microscopic observation.

Efflux of 6DOG from preloaded trypanosomes was inhibited by 2,4-dinitrofluorobenzene, 2-hydroxy-5-nitrobenzyl bromide and by n-ethylmaleimide. Fig. 49 shows the efflux of 6DOG over a 90 second period with and without 3mM inhibitor.

Greatest inhibition of efflux was found with 2-hydroxy-5-nitrobenzyl bromide in which after 30 seconds less than 10% of the total 6DOG had effluxed. This increased to 42.5% of the total at 90 seconds. The initial inhibitions of 2,4-dinitrofluorobenzene and N-ethyl-maleimide up to 10 seconds were similar; however after this point 2,4-dinitrofluorobenzene inhibited cells continued to efflux and after 90 seconds the total 6DOG entrapped in the trypanosomes was similar to that of the control experiment (no inhibitor). Efflux after

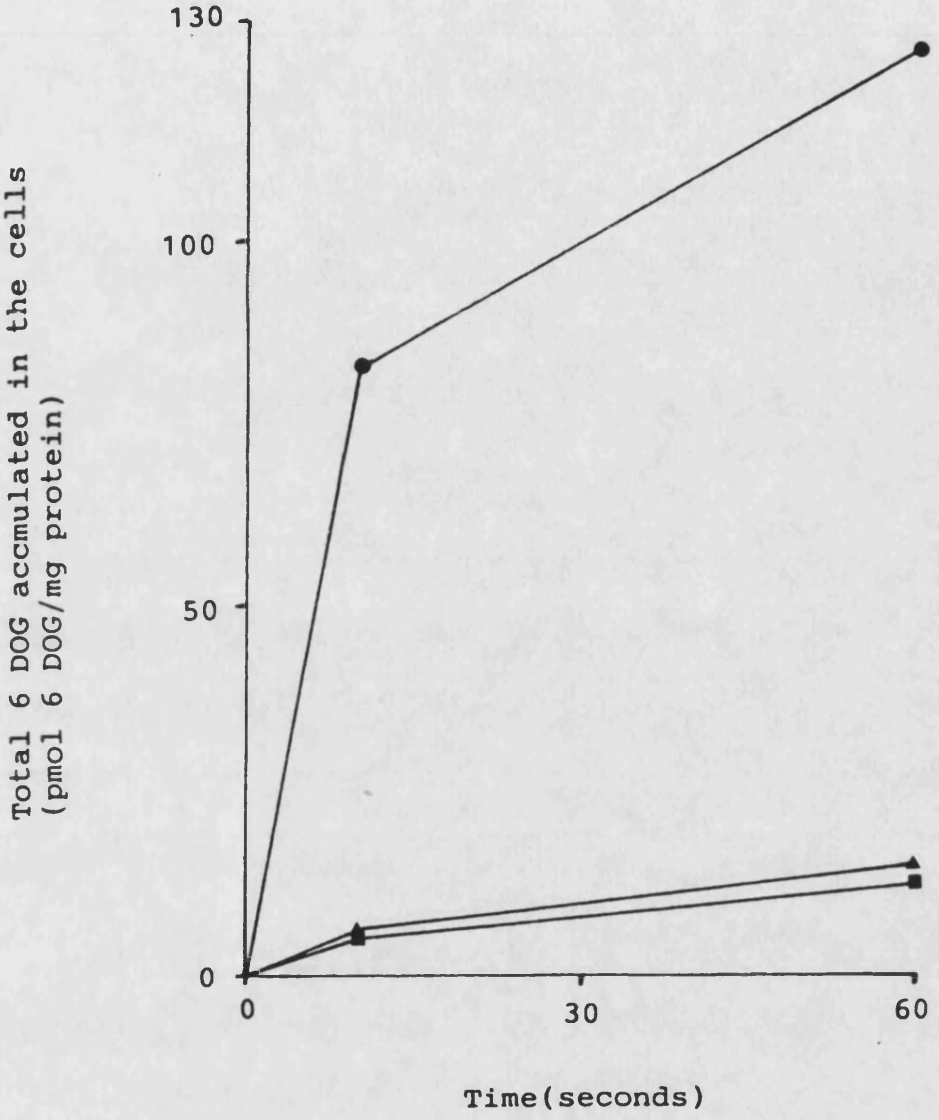


Fig. 48. Zero-trans 6DOG influx into trypanosomes in the absence of inhibitor (●), in the presence of 3mM 2-hydroxy-5-nitrobenzyl bromide after 1min preincubation (▲) and 10min preincubation (■) with the inhibitor.

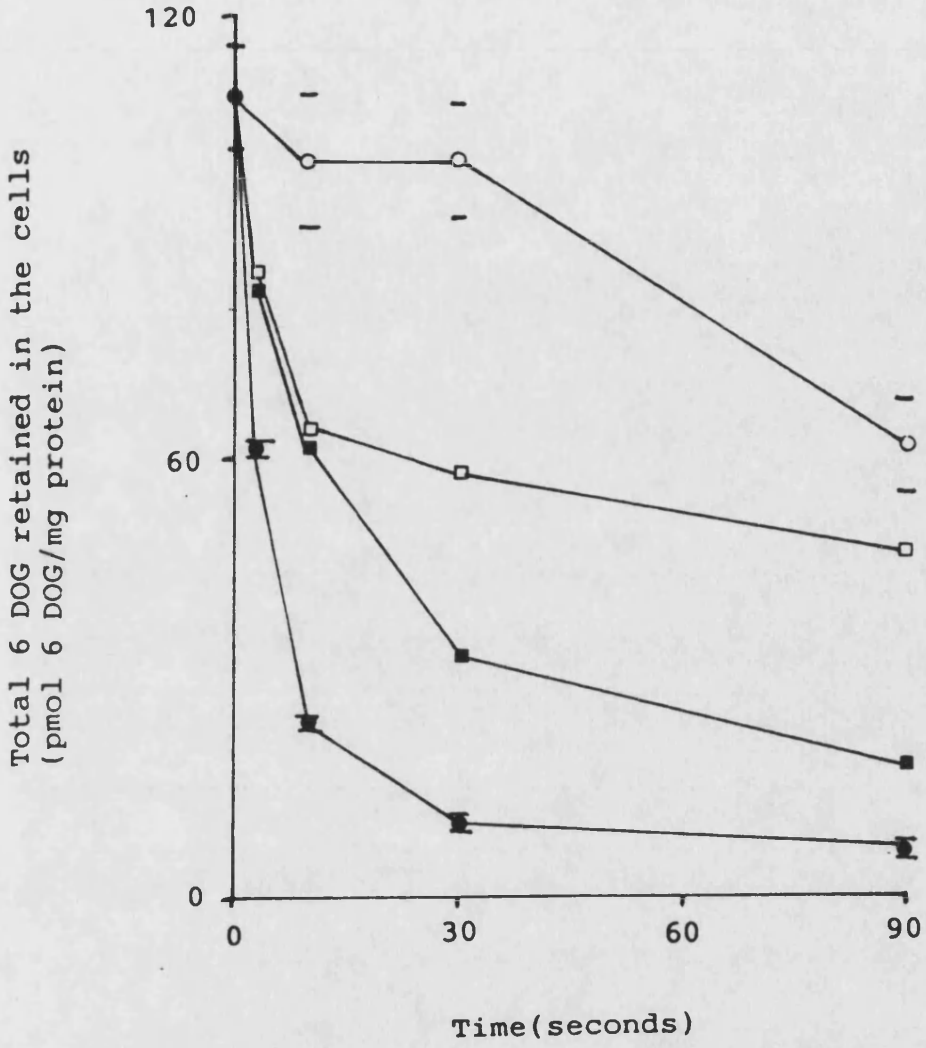


Fig. 49. 6DOG efflux from preloaded trypanosomes with time in the presence of no inhibitor (●), 3mM 2-hydroxy-5-nitrobenzyl bromide (○), 3mM N-ethylmaleimide (□) and 3mM 2,4-dinitrofluorobenzene (■).

10 seconds in the presence of N-ethylmaleimide slowed considerably and after 90 seconds the total 6DOG remaining in the trypanosomes was comparable to that of the cells treated with 2-hydroxy-5-nitrobenzyl bromide.

8.0 EFFECT OF DIHYDROLIPOAMIDE, NAD^+ AND PHENYL ARSINE OXIDE ON INFINITE-TRANS D-GLUCOSE TRANSPORT IN PLASMA MEMBRANE VESICLES

The kinetic constants of D-glucose infinite-trans-transport were determined using the Hanes-Woolf plot as fitted to the rate equation by the method of Cleland (1967). The best fit of the data used the unaltered values, *ie.* no leakage or dual transport systems could be detected.

Plasma membrane vesicles preloaded with 5mM NAD^+ and 100mM D-glucose exhibited infinite-trans D-glucose transport with a Km of 27.0±3.34mM and Vmax of 141.67±12.14nmol D-glucose/mg protein/min, fig.50. These values are in excess of those quoted previously for preloaded vesicles without 5mM NAD^+, of Km=15.66mM and Vmax=62.9nmol/mg protein/min.

Addition of dihydrolipoamide (1mM) to the NAD^+ and D-glucose preloaded vesicles reduced the Km to 22.1±2.56mM and the Vmax to 76.95±5.9nmol/mg protein/min, fig. 51.

Application of the Students T-test to the Kms for D-glucose transport in the (NAD^+ preloaded) plasma membrane vesicles, in the presence and absence of dihydrolipoamide produced a value of $P < 0.25$. This suggests that the Kms are significantly different and dihydro-lipoamide is altering the binding properties of the transporter. For absolute certainty, however, a value of $P < 0.05$ would be expected. It cannot therefore be

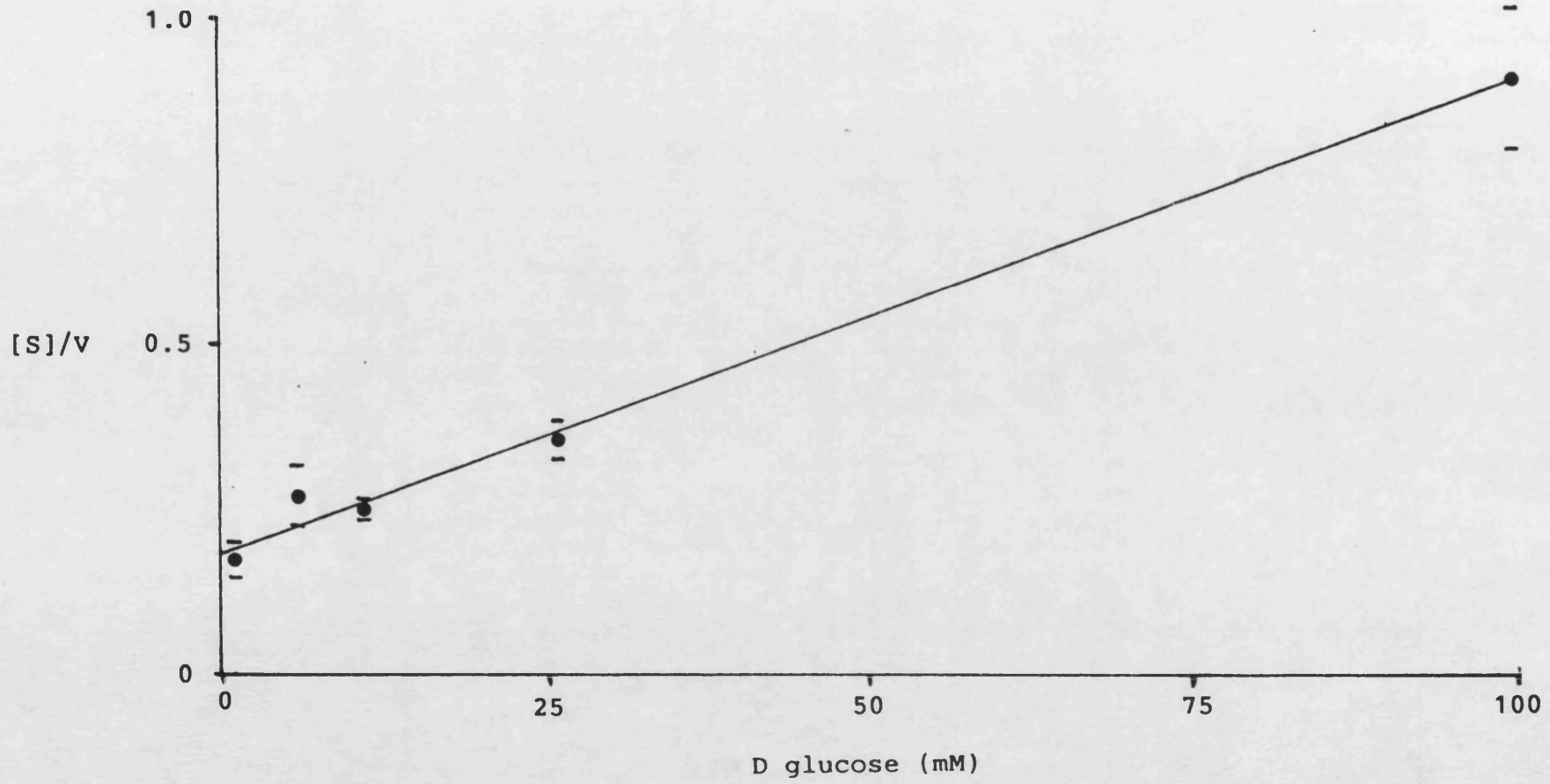


Fig. 50. Hanes plot to determine the Km and Vmax for infinite-trans D-glucose transport into plasma membrane vesicles preloaded with 100mM D-glucose and 5mM NAD^+.

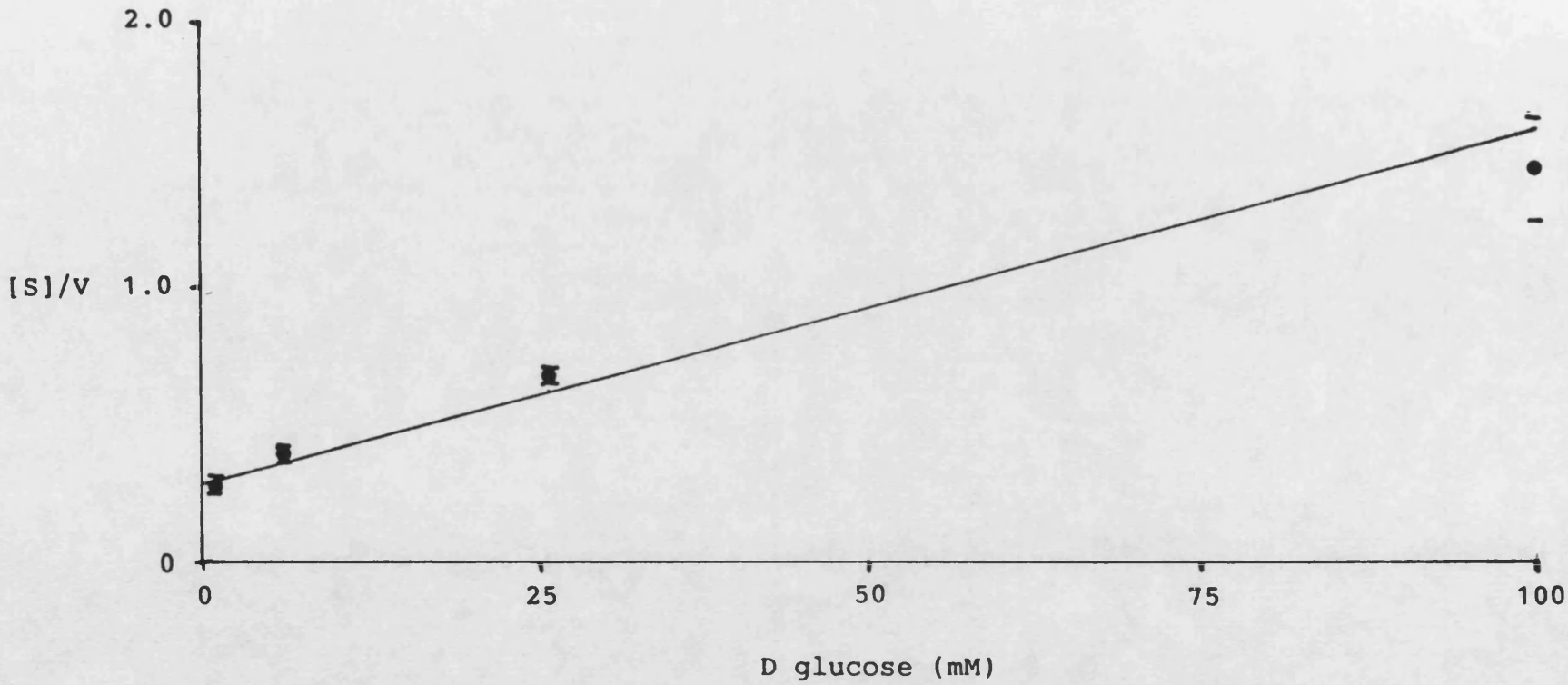


Fig. 51. Hanes plot to determine the Km and Vmax for infinite-trans D-glucose transport into plasma membrane vesicles preloaded with 5mM NAD^+ and 100mM D-glucose in the presence of 1mM dihydrolipoamide.

stated beyond doubt that the difference in Kms represent an alteration of D-glucose binding by the D-glucose transporter as a result of dihydrolipoamide.

When 200μM phenylarsine oxide replaced 1mM dihydrolipoamide the Km decreased to 13.87±2.24mM and the Vmax to 50.01±5.77nmol/mg protein/min, fig. 52, figures comparable to those obtained in the absence of 5mM NAD^+ preloading.

8.1 EFFECT OF DIHYDROLIPOAMIDE AND PHENYLARSINE OXIDE ON 6DOG TRANSPORT IN WHOLE VIABLE TRYPANOSOMES

The zero-trans influx transport of 6DOG into the trypanosomes was not inhibited to any significant degree (as compared to a control experiment with no inhibitor present) by either dihydrolipoamide (1mM) or phenylarsine oxide (100μM), fig. 53.

The efflux of 6DOG from the trypanosomes was inhibited by phenylarsine oxide and dihydrolipoamide, fig. 54. In the absence of inhibitor efflux was rapid over the first 10 seconds of incubation with over 75% of the total 6DOG released by the cells. This was followed by a slow phase of release reaching a minimum after 60 seconds incubation.

In the presence of dihydrolipoamide (1mM) and phenylarsine oxide (200μM, used singly in separate assays), less than 50% of the total 6DOG was released after 10 seconds incubation. With increasing time, dihydrolipoamide treated cells released the 6DOG, such that after 60

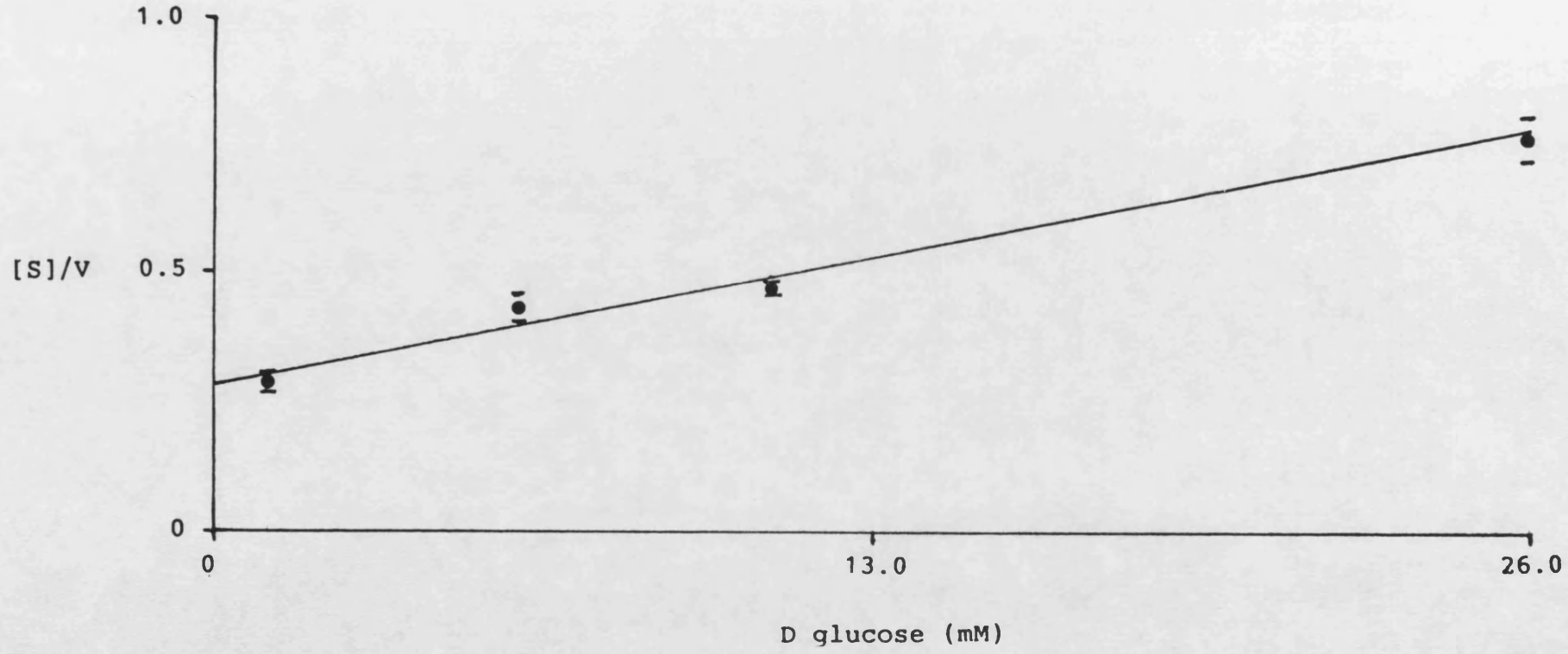


Fig. 52. Hanes plot to determine Km and Vmax for infinite-trans D-glucose transport into plasma membrane vesicles preloaded with 5mM NAD^+ and 100mM D-glucose in the presence of 200μM phenylarsine oxide.

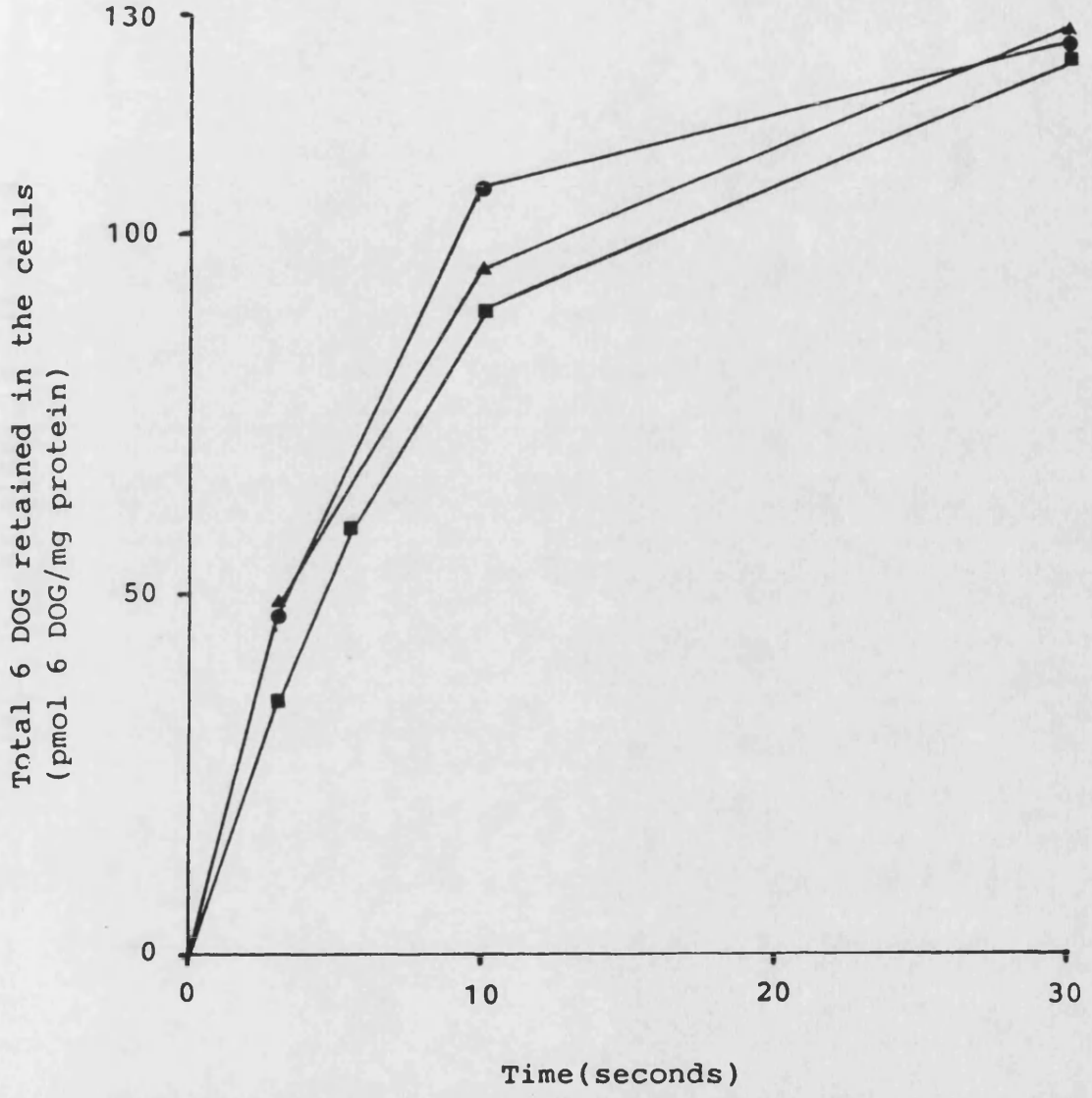


Fig. 53. Zero-trans 6DOG transport into trypanosomes in the absence of inhibitor (●), presence of 1mM dihydrolipoamide (■) and 100μM phenylarsine oxide (▲).

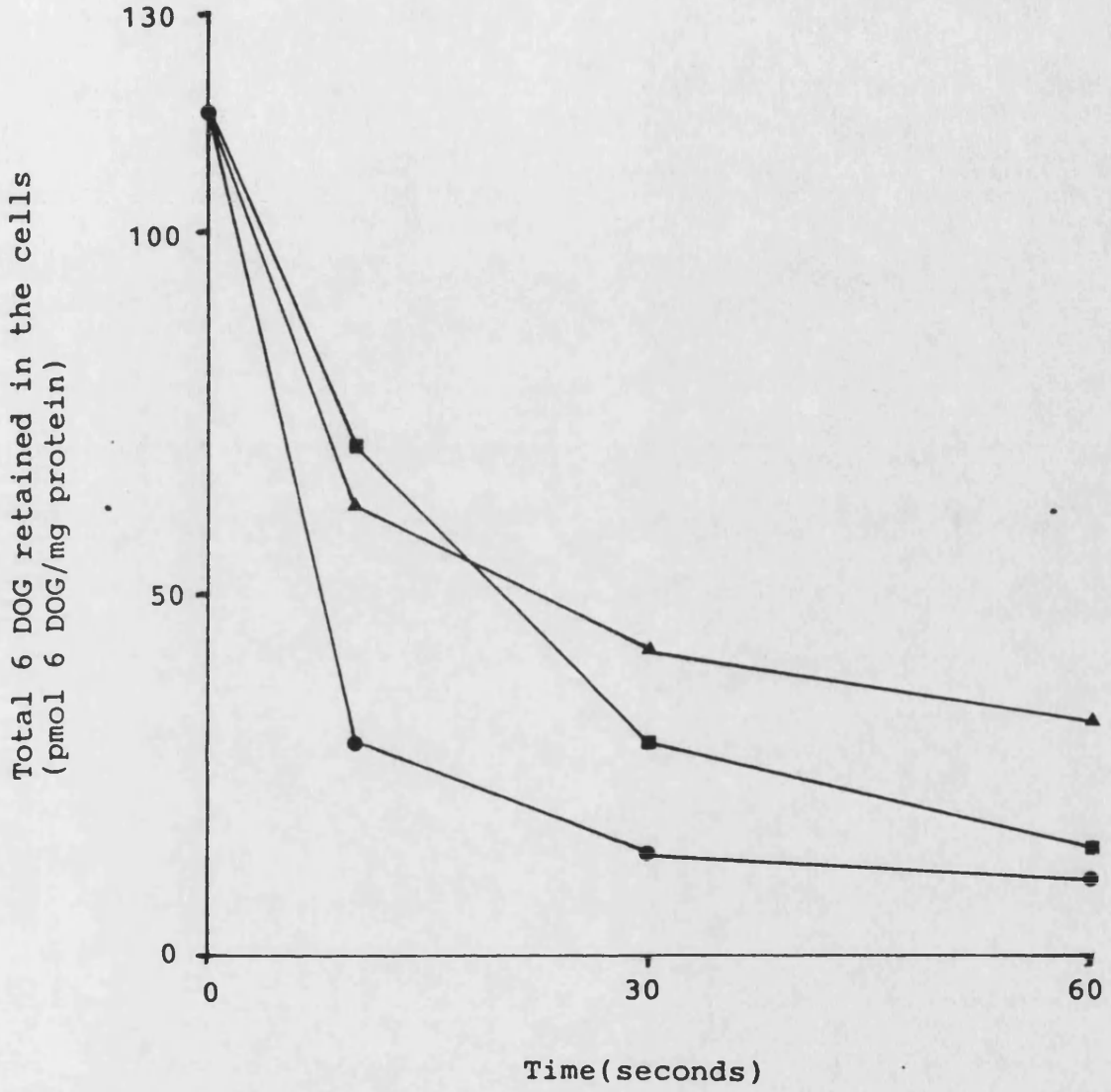


Fig. 54. Efflux of radiolabelled 6DOG from trypanosomes in the absence of inhibitor (●), presence of 1mM dihydrolipoamide (■) and 100μM phenylarsine oxide (▲).

seconds the total trapped 6DOG was comparable with that trapped in the absence of inhibitor. Phenylarsine oxide, however, inhibited the efflux of 6DOG such that after 60 seconds over three times as much 6DOG was retained in the trypanosomes compared to the control experiment, and the rate of efflux was approaching zero.

DISCUSSION

The mammalian bloodstream form of T.b.brucei is entirely dependent on D-glucose for its energy supply. It has no energy reserves and does not oxidise fatty acids or amino acids (Fairlamb, 1982). D-glucose transport has not been extensively studied in the organism though Gruenberg et al. (1978) stated that D-glucose transport was the rate limiting step in glycolysis. This observation presents the D-glucose transporter as an ideal site for the action of trypanocidal drugs.

The aim of this thesis was to study the isolated D-glucose transporter to avoid complications of cell metabolism, cell lysis and cell death. To this end the plasma membranes were prepared according to the method of Voorheis (1979) utilising a very tight fitting Dounce homogeniser. This disrupted the trypanosomes with one stroke, diminishing the loss of plasma membrane material through separation from the microtubular array, caused by repeated strokes of a looser fitting homogeniser (Voorheis et al., 1979).

The plasma membranes were the source of the D-glucose transporter and were treated to allow vesiculation and thus the study of the transporter in its native environment. More importantly the plasma membranes and any successfully treated plasma membranes represented a source of D-glucose transporters for solubilisation, ultimate purification, and reconstitution to study its activity.

1.0 PLASMA MEMBRANE PURITY

The marker enzymes for the cytoplasm, mitochondria, endoplasmic reticulum, glycosomes and plasma membrane were all found in varying degrees with the final plasma membrane pellet P4.

Cytosolic contamination, as represented by pyruvate kinase, was small with 2.5% of the total activity remaining with the final pellet. The activation of the enzyme by Triton X-100 may represent entrapment of the enzyme in the plasma membrane during the homogenisation process. Low cytosolic contamination was confirmed by malate dehydrogenase (90% of which is cytoplasmic in the bloodstream form (Opperdoes *et al.*, 1981)).

The remaining 10% of the malate dehydrogenase activity in the whole cells is associated with the mitochondrion (Opperdoes *et al.*, 1981). The low recovery of malate dehydrogenase (2% of the total enzyme activty), the complete absence of oligomycin sensitive Na^+K^+, Mg^{2+} stimulated ATPase and only 5.7% of the total glycerol-3-phosphate oxidase activity (both of which are mitochondrial markers, Opperdoes *et al.*, 1977b) in the final membranes suggests minimal mitochondrial contamination.

The enzyme α-glucosidase has been put forward as a plasma membrane marker enzyme (Steiger *et al.*, 1980), however only 13.2% of the total activty was recovered with the final plasma membrane pellet. Though co-purifying with the plasma membrane fraction in the

subcellular fractionation of Steiger et al. (1980), the enzyme could be released totally by the action of 0.1% (w/v) Triton X-100 (Steiger et al., 1980). This suggests that the enzyme is not strongly attached to the plasma membrane, ifit is attached at all, since 0.1% (w/v) Triton X-100 removed only approximately 33% of the total dihydrolipoamide dehydrogenase activity from the plasma membranes in this thesis. Its use as a definitive plasma membrane marker was therefore not possible.

D-glucose-6-phosphatase (10.5% of the total activity) was recovered in the plasma membranes. This enzyme has been suggested to be a marker enzyme for the trypanosome endoplasmic reticulum (Voorheis et al., 1979), however Steiger et al. (1980) have suggested that D-glucose-6-phosphatase activity is in fact attributable to acid phosphatase, which is located in the endoplasmic reticulum and flagellar pocket. The latter site would explain the D-glucose-6-phosphatase activity associated with the plasma membranes, with enzyme latency caused by disruption of the flagellar pocket during homogenisation.

Myokinase activity (6.8% of the total activity) was recovered with the final plasma membrane pellet P4. Two major sites have been suggested for myokinase; one in the mitochondria, and the second in the glycosome (Opperdoes et al., 1981). As already noted the mitochondrial marker enzymes suggest very little mitochondrial contamination of the plasma membranes,

and it therefore seems likely that the activity is associated with the glycosomal form.

Hexokinase, glycerol-3-phosphate dehydrogenase and phosphoglucoisomerase are all glycosomal enzyme markers (Opperdoes and Borst, 1977c). A maximum of 16.3% (phosphoglucoisomerase) of the total enzyme activity remained with the plasma membrane pellet at specific activities approximately the same as those of the homogenate. In all other enzyme markers assayed, excluding α-glucosidase and dihydrolipoamide dehydrogenase, specific activity in the plasma membranes was lower than the homogenate value.

Two explanations are possible for these observations. Glycosomes equilibrate at a sucrose gradient density of 1.23g/cm^3 (Opperdoes et al., 1977b,c) and plasma membranes at 1.243g/cm^3 (Voorheis et al., 1979), it is possible therefore that glycosomal contamination represents a cross over of the two bands. The second explanation is that some glycosomes or enzyme complexes are tightly bound to the plasma membranes, either naturally, or as a result of the homogenisation process.

In the former explanation the sucrose densities of 1.23g/cm^3 and 1.243g/cm^3 correspond approximately to 48.5% (w/v) sucrose and 51.3% (w/v) sucrose at 4°C, ie. a difference of 2.8% (w/v) sucrose. On a continuous gradient in the centrifuge tubes used this corresponds to a separation of the bands of approximately 1cm. No discernible band was seen 1cm above the plasma membrane band, and hence it is unlikely that an overlap of the

bands occurred.

The evidence suggests therefore that the latter explanation of some tightly bound glycosomes or enzyme complexes attached to the plasma membrane is more likely.

In the original purification of plasma membranes by Voorheis et al. (1979), hexokinase contamination was removed by raising the ionic strength of the homogenate by additions of potassium chloride. The major difference between the original Voorheis et al. (1979) technique and that reported here, is the initial removal of the hexokinase. This difference may be a facet of the different homogenisers employed and the hydrophobicity of the hexokinase, glycerol-3-phosphate dehydrogenase, phosphoglucoisomerase (Misset and Opperdoes, 1984); and/or the glycosomal contamination.

The activity of the plasma membrane marker, ouabain sensitive Na^+K^+ATPase as used by Voorheis et al. (1979) for the identification of the plasma membranes, proved extremely difficult to measure accurately and consistently. Steiger et al. (1980) were unable to measure any ouabain sensitive ATPase activity in the same organism and determined that ATP hydrolysis was a function of acid phosphatase activity carried over in the procedure. The co-purification of 10.5% of the total D-glucose-6-phosphatase activity with the plasma membranes explains the high background ATPase activity which made detection of small amounts of ouabain sensitive ATPase difficult to measure.

The plasma membranes are therefore essentially free of cytoplasmic and mitochondrial contamination but retain some contamination by glycosomal enzymes.

2.0 HEXOKINASE ACTIVITY AND FUNCTION

A significant amount of hexokinase activity was found to remain with the plasma membranes after the purification procedure.

It has been suggested that hexokinase is involved in the D-glucose transport system of yeast (Van Steveninck & Rothstein, 1965; Jaspers et al., 1975; Tijssen et al., 1984). Accumulation of phosphorylated 2-deoxy-D-glucose against a concentration gradient was balanced by a concomittant decrease in cellular ATP, orthophosphate and polyorthophosphate (Jaspers et al., 1975).

The hexokinase associated with the trypanosome plasma membranes demonstrated specificity for ATP and ADP as the phosphate donor in the presence of Mg^{2+}. Phosphoenolpyruvate, orthophosphate and pyrophosphate did not donate a phosphate group to the D-glucose. The Km for D-glucose with ATP as a phosphate donor was 48μM, a figure approximating to that of yeast hexokinase (0.1mM, Bergemyer, 1974a).

The ability of ADP to act as a phosphate donor can be explained by the presence of myokinase activity which is able (as shown in the marker enzyme assay) to catalyse the formation of ATP from ADP, which could then be used to phosphorylate D-glucose by hexokinase. The increased Km and decreased Vmax for the production of D-glucose-6-phosphate is a result of the reliance on myokinase to produce the ATP required. The specific activity of myokinase in the plasma membrane pellet

was 0.037μmoles ATP/mg protein/min compared to the specific activity of the hexokinase in the plasma membrane pellet P4 of 1.18μmoles D-glucose-6-phosphate/mg protein/min. Effectively, therefore, ATP would not be available in saturating quantities as it was in the hexokinase assay where ATP was the phosphate donor. It would therefore be expected that the kinetic constants Km and Vmax would be altered when ADP was the phosphate donor. Overall therefore the hexokinase activity would appear to be classical hexokinase activity.

Game et al. (1986) demonstrated that 1-deoxy-D-glucose was not phosphorylated to any extent by the trypanosome hexokinase in vitro. In vivo however, approximately 50% of the 1-deoxy-D-glucose transported into the cell was in the phosphorylated form (Game, 1988) indicating that 1-deoxy-D-glucose was a substrate for hexokinase or another phosphorylating process. Game (1988) also demonstrated that 6-deoxy-D-glucose was a substrate for the D-glucose transporter, though it cannot be phosphorylated in the C6 position. Transport of 6-deoxy-D-glucose into yeast lost the high affinity component of transport (Bisson & Fraenkel, 1983a) indicating that the kinase was required for high affinity transport but not by direct phosphorylation of the substrate as 6-deoxy-D-glucose cannot be phosphorylated in the C6 position. A similar process could occur in the trypanosome.

Hexokinase involvement in transport processes would require its presence in the cytosol or associated with the plasma membrane. Bisson and Fraenkel (1983b)

postulated that a small fraction of the yeast hexokinase was tightly bound to the plasma membrane or that there was a loose association with some membrane component that performs the recognition of the external sugar.

In the isolated trypanosome plasma membranes, the hexokinase associated with the plasma membranes appears to be the result of contamination by glycosomes or enzyme complexes. However, it cannot be ruled out that a small amount of hexokinase associated with the plasma membrane is involved in D-glucose transport.

3.0 DIHYDROLIPOAMIDE DEHYDROGENASE:ASSOCIATION WITH THE PLASMA MEMBRANE

Dihydrolipoamide dehydrogenase activity was found to co-purify with the purified plasma membranes, with some activation during the procedure.

Maximum, though not complete solubilisation of the enzyme activity was achieved at 0.5% (w/v) Triton X-100, a detergent concentration used for almost complete solubilisation of the erythrocyte membrane (Kasahara & Hinkle (1976); in contrast high salt had very little effect on releasing the enzyme. The enzyme was readily reconstituted into phosphatidyl choline vesicles by both detergent dilution and freeze/thaw/sonication procedures, with percentage total activity similar to the percentage of the total protein recovered in the proteoliposomes.

Significantly the reconstituted enzyme was firmly attached to the proteoliposomes, staying associated through further sonication, dilution and freezing and thawing procedures. The above suggests that the enzyme is attached to the plasma membrane by a hydrophobic interaction, and from the amount of Triton X-100 required to release it, either an integral membrane protein, or attached covalently to lipids embedded in the membrane, released by Triton X-100.

3.1 Possible roles for dihydrolipoamide dehydrogenase

It has been suggested that dithiol-disulphide

interchanges play a role in membrane related processes such as solute transport and energy transduction (Robillard & Konings, 1982). It is possible that these interractions are mediated through a co-factor such as lipoamide, and the dihydrolipoamide dehydrogenase, since the enzyme has now been found in the plasma membranes of E.coli (Owen et al., 1980), adipocyte plasma membranes (Karim, A.R., Holman, G.D. and Danson, M.J., unpublished data), and in thermoacidic archaebacterium (Smith et al., 1987). Preliminary experiments have demonstrated the presence of lipoic acid in T.b.brucei (Danson, M.J. and Stephenson, K., unpublished data), but not its location in the cell.

The exact role of dihydrolipoamide dehydrogenase in the plasma membranes, and lipoic acid (exact location unknown) in the trypanosome is not clear. Richarme (1985) demonstrated that lipoic acid deficient E.coli had a 60 to 80% reduction in protein dependent transport of ribose, galactose and maltose. In E.coli with lipoic acid present, arsenical (sodium arsenate and sodium arsenite) produced the same results as lipoic acid deficiency, but had no effect on lipoic acid deficient E.coli.

Further to the above, Richarme and Heine (1986) demonstrated that addition of dihydrolipoate and 3-acetyl NAD^+ to toluene/EDTA treated E.coli reactivated the galactose and maltose binding protein dependent transport lost during the toluene/EDTA treatment. No stimulation was observed in cells under the same conditions which were deficient in the galactose and maltose binding

protein dependent system.

D-glucose transport in the plasma membrane vesicles was affected by NAD^+, dihydrolipoamide and phenylarsine oxide. The Km of D-glucose transport in the absence of NAD^+ preloading in the vesicle was 15.66mM with Vmax 62.9nmol/mg protein/min. Preloading the vesicles with NAD^+ almost doubled the Km (27.0mM) and more than doubled the Vmax (141.67nmoles/mg protein/min). Addition of dihydrolipoamide decreased the Km to 22.1mM and Vmax to 76.95nmol D-glucose/mg protein/min. The significance of the change in Km is debatable as demonstrated by the Students T-test result (see Results Section 8.0), however, the Vmax is significantly reduced. Phenylarsine oxide decreased both the Km and the Vmax (to 13.87mM and 50.01nmol D-glucose/mg protein/min respectively). These values approximate to those of the vesicles without NAD^+ preloading.

The last result with phenylarsine oxide tends to suggest that the perturbation of the D-glucose transporter is not at the site of the D-glucose transporter but in a protein associated with it, since the NAD^+ effect was regulated by it. Experiments on whole cells did not prove conclusive. Inhibition of 6-deoxy-D-glucose efflux from preloaded trypanosomes was achieved by both dihydrolipoamide and phenylarsine oxide, the degree of which may represent the irreversible binding of the arsenical. In intact cells, however, no significant inhibition of zero-trans influx was observed.

Preliminary experiments on intact trypanosomes involving preincubation with arsenical reagents by

Arnold, M., Holman, G., and Eisenthal, R.S., demonstrated,
1) Phenylarsine oxide, and aminophenyldichloroarsine inhibited initial zero-trans entry of 6-deoxy-D-glucose by 50 to 80%, but acetylaminophenylarsine oxide proved ineffective.
2) Addition of 10mM D-glucose to arsenical incubation did not decrease the arsenical inhibition.
3) The decrease in D-glucose uptake by phenylarsine oxide was accompanied by a similar but less marked inactivation of the enzyme, dihydrolipoamide dehydrogenase.

None of the above is definitive proof that dihydrolipoamide dehydrogenase is associated with D-glucose transport in the trypanosomes. The effects of NAD^+, dihydrolipoamide and phenylarsine oxide could be explained, however, if they interfered with or altered the action of dihydrolipoamide dehydrogenase in the plasma membrane, rather than directly with the D-glucose transport transporter, by affecting a dithiol group essential for D-glucose transporter viability, not accessible to the chemical modifiers.

A second role for dihydrolipoamide dehydrogenase could be a proton pumping one. The action of dihydrolipoamide dehydrogenase producing NADH and a proton at the plasma membrane would simplify the pumping of a proton across the membrane. This could be important to the cells for two reasons.
i) It has been suggested that the activity of the lactose transporter in E.coli can be altered by changing the redox state of the carrier (Konings et al., 1982). It is possible, therefore, that dihydrolipoamide dehydrogenase

activity is connected to D-glucose transport indirectly by production of a proton gradient.

ii) If pyruvate is removed from the cell in the negatively charged state, the pumping out of the cell of a proton would balance the charge left inside the cell.

From the results in this thesis no definitive statement can be made on the proton pumping hypothesis. However, it was significant that preloading of the cells with NAD^+ which created a charge gradient between the inside and outside of the plasma membrane vesicles significantly altered the Km and Vmax of the D-glucose transport process. Addition of dihydrolipoamide would allow more proton pumping which may explain the decrease in Vmax and slight decrease in Km. On this basis however it is difficult to see why arsenical should remove the NAD^+ effect on D-glucose transport in the plasma membrane vesicles.

Van Schaftingen et al. (1987), found that the effect of melarsen oxide on trypanosomes was not attributable to inhibition of the glycolytic flux. Its role may therefore be in the inhibition of dihydrolipoamide dehydrogenase, and its possible roles in D-glucose transport, proton pumping or maintenance of the oxidation state of vicinal thiols.

4.0 PLASMA MEMBRANE TREATMENT

Alkali, calcium ions and freeze/thaw treatments of the plasma membranes were carried out to produce fragments or whole plasma membranes devoid of cytoskeleton and amenable to vesiculation without detergent extraction, hence maintaining the transporter in an environment as close to its natural one as possible.

Both the alkali and calcium ion treatments proved ineffective in providing an active D-glucose transport system, alkali treatment being particularly disruptive in its effect on the plasma membranes. This technique has been successfully applied to erythrocyte "ghosts" by Gorga *et al*. (1981) suggesting that the technique itself was not at fault but rather the disruption of the plasma membranes and the consequences of this on the D-glucose transporter environment and possible relevant protein:protein interractions, essential for activity. This view is supported by the inactivation of dihydrolipoamide dehydrogenase which would appear to be closely associated with the plasma membranes.

Calcium ion treatment of the plasma membranes also resulted in an inactivated D-glucose transporter. The process itself did not produce the required microtubule free plasma membranes as achieved by Dolan *et al*. (1986). Raising the calcium ion concentration to 1mM as used to depolymerise the microtubules of *Distigma proteus* (Murray, 1984) had no effect on microtubule release. A possible explanation is the "clumping" of the microtubules observed during the treatment. This

process would prevent calcium ions from reaching their point of action or allow only partial depolymerisation of the microtubules.

The freeze/thaw technique proved the only process to produce a functional D-glucose transporter. Unlike the alkali and calcium ion treatments, freezing and thawing did not require chemical action on the plasma membranes. It is possible, therefore, that the environment of the D-glucose transporter on the plasma membrane and/or protein:protein interractions were maintained such that the D-glucose transporter could function.

The major drawback of this process was its relative inefficiency in releasing the plasma membrane as vesicles. It was hoped that changing the ionic strength might release greater amounts of plasma membrane as vesicles, but more importantly lead to greater vesiculation and larger internal volumes to trap radiolabelled D-glucose. This was not the case and variation of ionic strength appeared to have little effect on the vesiculation process.

5.0 RECONSTITUTION OF THE ERYTHROCYTE AND TRYPANOSOME PLASMA MEMBRANE PROTEINS

5.1 Detergent extract reconstitution

The reconstitution of the membrane proteins from the trypanosome and erythrocyte plasma membranes was undertaken using a wide variety of detergents and reconstitution techniques, and two methods of assay, zero-trans and infinite-trans sugar transport.

Detergent dialysis of erythrocyte n-octyl-β-D-glucopyranoside extracts and freeze/thaw reconstitution of erythrocyte membranes extracted with n-octyl-β-D-glucopyranoside (removed by dialysis), Zwittergent 14 and Triton X-100 all proved successful in reconstituting the erythrocyte D-glucose transporter. These results demonstrate the reconstitution procedures themselves were capable of reconstituting membrane proteins, and in a functional state.

The results obtained with the erythrocytes could not be repeated with the trypanosome plasma membranes. Trypanosome plasma membrane protein and active dihydrolipoamide dehydrogenase were found associated with proteoliposomes formed by each reconstitution procedure, however, no D-glucose transport was found. Since the techniques employed had proved successful on the erythrocyte D-glucose transporter it would appear that inactivation of the D-glucose transporter was occurring at some point in the isolation of the plasma membranes or during the reconstitution procedure.

Inactivation during plasma membrane isolation is not credible because vesicles from the freeze/thaw treated plasma membranes demonstrated D-glucose transport; therefore inactivation was occurring during solubilisation and reconstitution.

The type of detergent and concentration have proved critical for effective reconstitution of membrane proteins. Banejee et al. (1977) demonstrated that preparations of the Pi/OH^- transporter of bovine heart mitochondrion were variable with cholate and deoxycholate, but reproducible and stable when extracted with n-octyl-β-D-glucopyranoside and Triton X-100. Similarly, Navarette and Serrano (1983) demonstrated that only Zwittergent 14 and lysolecithin effectively solubilised oat root ATPase, but only lysolecithin produced a stable enzyme during the purification and reconstitution procedure (Serrano, 1984).

Solubilisation of the trypanosome plasma membranes utilised n-octyl-β-D-glucopyranoside, Triton X-100, sodium cholate, Zwittergent 14, decanoyl-N-methyl-glucamid and L-α-lysolecithin to provide detergents that were non-ionic (high and low critical micelle concentration), ionic, charged but electrically neutral, and lipid-like. In no case was D-glucose transport activity found in the reconstituted extracts.

Apart from reconstitution technique and detergent several other factors have proved crucial to effective reconstitution of membrane proteins. These include lipid type used in the reconstitution process, lipid to protein

ratios and stabilising chemicals such as glycerol.

The lipid used predominantly in the reconstitution process was L-α-lecithin derived from soybeans. This lipid source was a mixture of lipids containing only 21% phosphatidyl choline and was therefore more likely to contain factors required for D-glucose transporter viability than pure lipid sources, *ie.* the pure egg phosphatidyl choline. In the reconstitution of *E.coli* lactose transporter Newman and Wilson (1980) demonstrated a requirement for *E.coli* lipid in the reconstitution process for lactose transporter viability. The reconstitution of the trypanosome membrane proteins in trypanosome lipid by the detergent dilution procedure was ineffective, and use of the trypanosome lipid discontinued because of the difficulty in obtaining the large quantities required, and the problem of sonicating the lipid to clarity for efficient reconstitution and replication.

In the reconstitution of other membrane proteins several other factors have proved important for membrane protein function and integrity. Calcium or magnesium ions were required for the reconstitution of adipocyte D-glucose transporter activity (Carter-Su *et al.*, 1980), protein:lipid ratios of 1:45 were required for optimal activity in the reconstitution of the purified Na^+K^+ATPase of *Electrophorus electricans* and *Squalus acanthias* (Hokin and Dixon, 1979), and the presence of 20% (v/v) glycerol in buffers for optimum stability of oat root ATPase during solubilisation and reconstitution (Vara and Serrano, 1982).

Apart from altering the buffer system of the reconstitution process from Tris/HCl to phosphate buffer (hence avoiding pH change with temperature), no attempt was made to vary the lipid:protein ratios, add divalent metal ions, or add stabilising compounds such as glycerol. It may well prove that alterations or addition of these factors will prove crucial to effective reconstitution of a viable trypanosome D-glucose transporter.

5.2 Non-detergent extract reconstitution

Non-detergent extract reconstitution was applied to plasma membrane vesicles which had a functional D-glucose transporter present. This had two advantages: firstly it avoided solubilisation with detergents (a possible cause of inactivation of the D-glucose transporter), and secondly provided a much larger internal volume to allow kinetic studies without the pre-requisite preloading of the vesicles with D-glucose.

Reconstitution without detergents has been applied successfully to erythrocyte membranes by Wheeler (1986) and to yeast plasma membranes by Franzusoff and Cirillo (1983b), and Ongjoco et al. (1987). In the case of yeast plasma membranes, D-glucose transport in plasma membrane vesicles was increased 10-fold by the reconstitution process (Franzusoff and Cirillo, 1983b).

The proteoliposomes derived from the reconstitution of the trypanosome plasma membrane vesicles did not demonstrate the overshoot phenomenom of D-glucose transport, but rather the leakage of D-glucose. A similar effect

was noted for the transport of L-glucose in the reconstituted yeast plasma membranes of Franzusoff and Cirillo (1983b). In the yeast proteoliposome case, however, the L-glucose "leakage" was only a fraction of D-glucose transport activity, hence the process was useful for measuring D-glucose transport activity.

The trypanosome plasma membrane vesicles before reconstitution demonstrated a maximum infinite-trans D-glucose accumulation of 1.69nmoles D-glucose/mg protein; after two minutes incubation. A transport rate of this magnitude would be masked by the leakage component of the reconstituted proteoliposomes (assuming no increase in transport rate). It is probable, therefore, that the D-glucose transporter was functional, but its activity was not detected under the conditions used. There is no reason to suppose that the freeze/thaw conditions used to make the proteoliposomes should inactivate the D-glucose transporter.

A similar D-glucose infinite-trans D-glucose transport time course was obtained with proteoliposomes formed from the fusion of plasma membrane vesicles and L-α-lecithin vesicles by L-α-lysolecithin. This technique has proved successful in reconstituting cytochrome oxidase (Eytan et al., 1975), however, as stated previously for the direct membrane protein incorporation by the freeze/thaw/sonication method, the transport component of D-glucose influx could easily be masked by the "leakage" component of D-glucose influx. It is also possible that the presence of L-α-lysolecithin, though lipid-like

in its structure , inactivated the D-glucose transporter, and no facilitated transport of D-glucose was occurring at all.

6.0 SUGAR SPECIFICITY AND KINETICS OF THE PLASMA MEMBRANE VESICLE D-GLUCOSE TRANSPORTER

The availability of plasma membrane vesicles with an active D-glucose transporter allowed the study of the transport of metabolisable sugars, i.e. D-glucose and 1-deoxy-D-glucose, and the inhibition of transport of sugars by other metabolisable or partially metabolisable compounds.

The Km's of the substrates D-glucose (15.66mM), 1-deoxy-D-glucose (17.26mM) and 6-deoxy-D-glucose (16.16mM) were considerably greater than those values obtained in intact trypanosomes, D-glucose 0.387mM, 6DOG 1.54mM (Game, 1988) and 1DOG 4.03mM (Game et al., 1986). An apparent increase in Km in vesicle systems has been found in reconstituted yeast and erythrocyte D-glucose transporters. Ongjoco et al. (1987) reconstituted the yeast D-glucose transporter and found the Km was 8.0mM, considerably higher than the high affinity transporter (Km = 0.5 to 1.0mM), but close to the Km of the low affinity D-glucose transporter (Km = 10 to 50mM). In the reconstitution of the band 3 of erythrocytes by Shelton and Langdon (1983), the Km of the D-glucose transporter was 15mM, a figure approximating to that found in erythrocyte "ghosts" (Km = 10mM) and erythrocyte membrane vesicles (Km = 10.9mM) by Taverna and Langdon (1973), all of which exceed the Km of 1.6mM in intact erythrocytes (Lacko et al., 1972).

The Vmax for D-glucose transport into the plasma

membrane vesicles was 62.9nmol/mg protein/min, similar to that for 6-deoxy-D-glucose transport of 53.67nmol/mg protein/min. These values can be compared with the Vmax of 6-deoxy-D-glucose transport in intact trypanosomes as determined by Game (1988), by conversion of the plasma membrane vesicle system Vmax units into $mMsec^{-1}$ (as used by Game, 1988). The Vmax in intact trypanosomes of 6-deoxy-D-glucose transport is $0.401mMsec^{-1}$ (Game, 1988), which compares favourably with $0.21mMsec^{-1}$ and $0.18mMsec^{-1}$ for D-glucose and 6-deoxy-D-glucose transport in the vesicle system (taking the internal vesicle volume to be 5.07µl/mg protein as determined in this thesis). In the reverse calculation, *ie.* converting the Vmax for 6-deoxy-D-glucose in the trypanosomes as determined by Game (1988) into nmoles 6DOG/mg protein/min, the Vmax of $0.401mMsec^{-1}$ becomes 33.1nmoles 6DOG/mg protein/min (taking the internal volume of the trypanosomes available to substrate to be 1.2µl per 1×10^8 cells (Game, 1988), and the total protein in 1×10^8 cells to be 0.872mg as determined by the author). These Vmax values are similar suggesting that the D-glucose transporter in the plasma membranes is functioning as it would in the intact trypanosomes.

The technique of using plasma membrane vesicles to determine D-glucose flux is not widespread and comparisons are therefore difficult. In erythrocyte membrane vesicles produced by endocytosis of erythrocyte "ghosts", the maximum rate of D-glucose accumulation was almost identical to that of erythrocytes and

erythrocyte "ghosts" (Taverna & Langdon, 1973), a similar finding to that in the trypansomes.

The higher Km than expected suggests that either the isolation of the plasma membranes and their subsequent vesiculation altered the D-glucose transporter substrate binding, or the D-glucose transporter binding is only optimum in an intact cell environment. Based on the results presented in this thesis no definitive answer can be provided ; however, in reconstituted erythrocyte "ghosts" Wheeler (1986) suggested that ATP affected D-glucose transport by altering the membrane structure. It is possible,therefore, that intracellular events or components could determine the Km of the trypanosome D-glucose transporter.

6.1 Comparison of inhibition of 6DOG transport in intact trypanosomes and plasma membrane vesicles

The inhibition of 6DOG transport in the plasma membrane vesicles allows a comparison of the D-glucose transporter inhibition characteristics with intact trypanosomes as determined by Game (1988). It should be noted that the values of the % inhibitions were carried out with an inhibitor concentration of 25mM. This concentration will be above or below the Ki for each individual inhibitor, resulting in different saturations of the D-glucose transporter by each inhibitor. This will effect the competition with the 6-deoxy-D-glucose substrate (1mM). Having said this one would expect that substrates with similar Kis in the inhibition of 6-deoxy-D-glucose transport in intact trypanosomes, would produce

similar % inhibitions of 6-deoxy-D-glucose transport in the plasma membrane vesicles.

Plasma membrane vesicle 6DOG transport was inhibited to a similar extent by D-galactose and D-mannose, 33% and 32% respectively, whereas in intact trypanosomes the Ki's of the two sugars were >250mM and 0.67mM respectively. In intact trypanosomes 3-O-methyl-D-glucose and 5-thio-D-glucose demonstrated Ki's of 15.38mM and 11.67mM respectively, considerably greater than the Ki's of D-glucose at 0.9mM. Inhibition of the plasma membrane vesicle D-glucose transporter by the three sugars was in the range 41% (5-thio-D-glucose) to 58.4% (D-glucose), but not the several orders of magnitude seen in the intact cells.

A similar trend was observed with D-glucosamine and N-acetyl-D-glucosamine, both of which inhibited intact cells with Ki's of 21.34mM and 11.11mM respectively, figures comparable to the Ki's of 3-O-methyl-D-glucose and 5-thio-D-glucose. The inhibition of 6DOG transport in the plasma membrane vesicles by D-glucosamine and N-acetyl-glucosamine were 41.8% and 42.7%, respectively, values approximating to that achieved by 3-O-methyl-D-glucose and 5-thio-D-glucose. In this case the inhibition characteristics of the two systems are similar. However, the inhibitions in the vesicle system are approximately equal in value to that of D-glucose inhibition, a situation patently not the case in the intact cells where D-glucose is a far more potent inhibitor of 6DOG transport.

Glycerol did not inhibit D-glucose transport

in the plasma membrane vesicles consistent with Games' (1988) observation in intact cells. This result is in contrast to that of Ruff and Read (1974) who demonstrated inhibition of sugar uptake by glycerol in T.equiperdum, and Southworth and Read (1969 and 1970) who found a similar effect in T.b.gambiense. Game (1988) suggested that these findings were a result of long incubation and processing times employed in these studies. Under the conditions employed only phosphorylated metabolites would be retained in the cells depleting ATP levels by the action of glycerol kinase (Opperdoes and Borst, 1977c). Phosphorylation of other substrates would not occur and this apparent inhibition of substrates would not be observed.

Both maltose and D-fructose inhibited the D-glucose transporter in the intact cells and plasma membrane vesicles. Maltose can be considered a C4 modified D-glucose molecule. In the intact cells, the Ki for maltose was 16.48mM, considerably lower than that for D-galactose (Ki = >250mM), a situation reversed in the plasma membrane vesicles, where maltose was a poorer inhibitor than D-galactose.

D-fructose, though a sugar metabolised by the cell, was not as efficient an inhibitor as any of the other sugars (inhibition of 25.5% in the plasma membrane vesicles). This may well be a result of a requirement for a pyranose ring structure for sugar transport, a configuration that can only be resembled by D-fructose in an open chain configuration.

Phloridzin which contains a phloretin moiety on the C1 position of D-glucose was a potent inhibitor of transport in the intact cells and achieved 15.9% inhibition of transport in the plasma membrane vesicles. Though this figure is low compared to the other sugars tested, the phloridzin concentration (2.2mM) was less than one tenth that of the other inhibitors because of its low solubility. Phloridzin was therefore the most effective inhibitor tested. Game (1988) has suggested that the inhibition is a result of the phloretin interracting with the transporter rather than the D-glucose portion of the compound.

6.2 D-glucose transporter binding requirement

The number of inhibitors tested on the plasma membrane vesicle system does not allow a comprehensive comparison of the D-glucose transporter with other D-glucose transporters, or a definitive analysis of binding requirements at each carbon position of D-glucose, however several observations can be made.

C2 analogues of D-glucose, D-mannose, D-glucosamine and N-acetyl-D-glucosamine all gave greater than 26.6% inhibition of D-glucose transport and greater than 32% inhibition of 6DOG transport. This suggests there is some spatial freedom around the C2 position, a freedom also found in the erythrocyte and adipocyte D-glucose transporters (Barrett et al., 1973; Holman et al., 1981) and intact trypanosomes (Game, 1988), but markedly different from the intestinal sodium cotransport system

(Crane, 1960), which requires an absolute D-glucose configuration at C2.

The C3 analogue tested, 3-O-methyl-D-glucose, was a strong inhibitor of D-glucose transport in the vesicles, but was a much poorer inhibitor in the intact trypanosomes (Game, 1988). The vesicle system is acting in a similar manner to the erythrocyte and adipocyte D-glucose transporters where 3-O-methyl-D-glucose is rapidly transported (Whitesell & Glieman, 1979; Glieman et al., 1972). At C3 there is spatial freedom not found in the intact trypanosome transporter.

D-galactose and maltose (C4) both inhibited D-glucose transport into the vesicles. D-galactose is also an inhibitor of the human erythrocyte transporter (Barrett et al., 1973) and in this respect the vesicle transporter is acting more like the erythrocyte transporter than the intact trypanosome transporter, where D-galactose is a poor inhibitor. It appears, therfore, that the requirement for an hydroxyl group at C4 has been decreased in the vesicle transporter.

The C5 analogue, 5-thio-D-glucose, was a good inhibitor of D-glucose transport equivalent to 1DOG and 6DOG in magnitude. This suggests that the ring oxygen is not vital to binding of D-glucose to the transporter, an observation also made in intact trypanosomes (Game, 1988).

Only 1DOG and 6DOG were tested for C1 and C6 requirements. Their inhibition of D-glucose transport was equivalent to D-glucose and thus the loss of hydroxyl

groups at C1 and C6 have not affected binding and transport. A similar observation was made in the intact cells (Game, 1988).

7.0 EFFECT OF CHEMICAL MODIFYING INHIBITORS ON THE PLASMA MEMBRANE D-GLUCOSE TRANSPORTER

Inhibition of D-glucose transport in the plasma membrane vesicles was achieved by 2,4-dinitrofluorobenzene, N-ethylmaleimide and 2-hydroxy-5-nitrobenzyl bromide.

N-ethylmaleimide specifically labels reactive sulphydryl groups and non-specifically some α-amino groups and imidazole rings, and 2,4-dinitrofluorobenzene labels N terminal amino groups and the $-NH_2$ of lysine. Neither inhibitor achieved greater than 34% inhibition of D-glucose transport at inhibitor concentrations up to 5mM. The D-glucose transporter would therefore not appear to contain any amino groups or sulphydryl groups (accessible to the modifying agents) involved in the transport process either in the active site or maintaining protein configuration.

The inhibitor 2-hydroxy-5-nitrobenzyl bromide which is specific for tryptophan residues was a superior inhibitor to the two inhibitors mentioned above. At 2mM inhibitor 46% inhibition of D-glucose was achieved, rising to 69.5% inhibition at 5mM inhibitor. If the freeze/thaw vesiculation process produces vesicles with a 50:50 orientation of inside/out to outside/in vesicles the 46% inhibition represents almost total inhibition of one set of the vesicle population. Inhibition rising to 69.5% at 5mM inhibitor represents inhibition of the external and internal sites as inhibitor crosses the vesicle bilayer.

Inhibition of the intact trypanosome D-glucose transporter was total when preincubated with the 2-hydroxy-5-nitrobenzyl bromide and efflux inhibited such that after 90 seconds 57.5% of the total label was trapped in the trypanosomes. This second assay measures D-glucose transport in trypanosomes with no preincubation in the presence of inhibitor; rather, inhibitor is added to the cells upon addition of diluting buffer. The almost immediate inhibition of efflux upon inhibitor addition and the inhibition characteristics in the vesicle system suggest that the inhibition is of a tryptophan residue on the external site of the D-glucose transporter. The association of enzyme activity with tryptophan residues has been noted in enzymes such as lysosyme (Witkop, 1968) from hen's egg in which the action of 2-hydroxy-5-nitrobenzyl bromide reduced the enzymes activity by 90% when alkylating tryptophan residue 6.

The inhibition of efflux of 6DOG from the intact trypanosomes by N-ethylmaleimide though comparable to that of 2-hydroxy-5-nitrobenzyl bromide, did not produce the same result in the plasma membrane vesicle system. Its activity cannot, therefore, be definitively attributed to a chemical modification of the D-glucose transporter.

8.0 FUTURE WORK

The future of this work lies in the effective solubilisation and reconstitution of the D-glucose transporter, involving a stepwise process.

1) The removal of the cytoskeleton from the plasma membranes needs to be perfected to provide plasma membranes more amenable to vesiculation and in large enough quantities to allow transport studies. These plasma membrane vesicles could then be tested for the effect of protective agents such as mercaptoethanol, D-glucose and glycerol, or addition of lipids or cholesterol to optimise D-glucose transport activity.

2) Based on the specificity studies of Game (1988) and the inhibition studies in the plasma membrane vesicle system, carbon C2 of the D-glucose ring has the spatial freedom for modification. A label based on the spatial freedom of C2 would provide a means of detecting D-glucose binding by the D-glucose transporter through all treatments, and this would aid identification of harmful or helpful conditions or chemical agents. This would be particularly useful in the reconstitution of the D-glucose transporter, identifying whether solubilisation by detergent, detergent removal, or reconstitution involving sonication and/or freeze/thaw procedures are harmful.

3) The role of dihydrolipoamide dehydrogenase requires further investigation. It is very difficult to design experiments that conclusively link it to a role in D-glucose transport. However, its attachment to the

plasma membrane could be investigated particularly to determine if it is an integral membrane protein or not. The enzymes possible role as a proton pump could be investigated using the reconstituted protein, which was successfully reconstituted in L-α-lecithin vesicles.

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Dihydrolipoamide dehydrogenase from *Trypanosoma brucei*

Characterization and cellular location

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Dihydrolipoamide dehydrogenase has been discovered in the bloodstream form of the eukaryotic African parasite, *Trypanosoma brucei*. The enzyme catalysed the stoichiometric oxidation of dihydrolipoamide by NAD^+ and exhibited a hyperbolic dependence of catalytic activity on the concentrations of both dihydrolipoamide and NAD^+. Chemical modification with the tervalent arsenical reagent *p*-aminophenyldichloroarsine indicates the involvement in catalysis of a reversibly reducible disulphide bond. Plasma-membrane sheets were purified from *T. brucei*, and it was shown that virtually all the dihydrolipoamide dehydrogenase remained closely associated with this membrane preparation. *T. brucei* apparently lacks the 2-oxoacid dehydrogenase multienzyme complexes of which dihydrolipoamide dehydrogenase is usually an integral component. In the context of this absence, the possible function of trypanosomal dihydrolipoamide dehydrogenase is discussed, with particular reference to its cellular location in the plasma membrane.

INTRODUCTION

Dihydrolipoamide dehydrogenase (EC 1.8.1.4) catalyses the NAD^+-dependent oxidation of dihydrolipoamide [reviewed by Williams (1976)]:

$$\text{Dihydrolipoamide} + NAD^+ \rightleftharpoons \text{lipoamide} + NADH + H^+$$

The enzyme fulfils this function in the pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase and branched-chain 2-oxo acid dehydrogenase complexes and is an integral component of each of these multienzyme structures. However, we have reported previously (Danson, 1984; Danson *et al.*, 1984, 1986) that, although archaebacteria do not possess these 2-oxo acid dehydrogenase complexes, they do have a dihydrolipoamide dehydrogenase, although its function *in vivo* remains unclear.

In the present paper we report our investigation of dihydrolipoamide dehydrogenase in the eukaryotic African parasite, *Trypanosoma brucei*. The bloodstream form of this trypanosome is totally dependent on glycolysis for ATP production, as it has no lipid or carbohydrate reserves and the mitochondrion does not possess a competent citric acid cycle (reviewed by Fairlamb, 1982). Thus it does not have the NAD-linked 2-oxo acid dehydrogenase complexes, and under aerobic conditions glucose is metabolized almost completely to pyruvate, approx. 2 mol of pyruvate being produced per mol of glucose (Fairlamb, 1982). *T. brucei* therefore provides us with a eukaryotic organism in which to look for the presence of dihydrolipoamide dehydrogenase in the absence of the 2-oxo acid dehydrogenase complexes. We not only report its presence and enzymological characteristics, but also show that it is associated with the plasma membrane of the trypanosome. The functional significance of its presence and cellular location is discussed.

EXPERIMENTAL

Materials

Chemicals used were of analytical grade or the finest grade commercially available. NAD^+, $NADP^+$ and NADH were from Boehringer, Mannheim, Germany; Nbs_2 and type II-S soya-bean L-α-phosphatidylcholine were from Sigma Chemical Co., Poole, Dorset, U.K.; DL-lipoamide was from BDH Chemicals, Poole, Dorset, U.K.; and high-purity Triton X-100 was from Pierce and Warriner (U.K.), Chester, U.K. DL-Dihydrolipoamide was prepared by the reduction of DL-lipoamide with $NaBH_4$ (Reed *et al.*, 1958), and *p*-aminophenyldichloroarsine ($H_2NPhAsCl_2$) was synthesized as described by Stevenson *et al.* (1978).

Growth and preparation of cells

Cells of the long slender form of *T. brucei* were isolated from the blood of Wistar rats infected with 1×10^7–3×10^7 cells of strain MITat 1.1 by intraperitoneal injection as described by Eisenthal & Panes (1985) and were further purified by chromatography on DEAE-cellulose (Lanham & Godfrey, 1970).

Cell extraction

Cells (approx. 0.1 g wet wt.) were resuspended in 1 ml of 50 mM-potassium phosphate (pH 7.0)/2 mM-EDTA. The suspension was sonicated at 0 °C for 3 × 30 s at 40 W with a 3 mm probe on an ultrasonic disintegrator; the cell debris was removed by centrifugation for 3 min at 10000 *g* ($r_{av.}$ 5.5 cm) and the supernatant stored at 4 °C.

Enzyme assays

Dihydrolipoamide dehydrogenase was assayed at 30 °C in 50 mM-potassium phosphate (pH 7.0)/2 mM-EDTA containing 0.4 mM-dihydrolipoamide and 1 mM-NAD^+. The reaction, in a final volume of 1 ml, was

Abbreviation used: $H_2NPhAsCl_2$, *p*-aminophenyldichloroarsine.
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started with enzyme and its progress was monitored by the increase in A_{340}.

Malate dehydrogenase was assayed at 30 °C in 50 mM-potassium phosphate (pH 7.0)/2 mM-EDTA containing 0.1 mM-NADH and 0.2 mM-oxaloacetate. The reaction, in a final volume of 1 ml, was started with enzyme and its progress was monitored by the decrease in A_{340}.

Chemical modification with $H_2NPhAsCl_2$

Modification of dihydrolipoamide dehydrogenase with 0.5 mM-$H_2NPhAsCl_2$ was carried out at 4 °C in 50 mM-potassium phosphate (pH 7.0)/2 mM-EDTA and in the presence and absence of 50 μM-NADH.

Preparation of trypanosomal plasma membrane

Sheets of plasma membrane from *T. brucei* were prepared as described by Voorheis *et al.* (1979). In outline, this procedure was as follows. Fresh trypanosomal cells were resuspended in 10 ml of Tes buffer (2 mM-Tes, pH 7.5, containing 150 mM-KCl, 1 mM-EGTA, 1 mM-2-mercaptoethanol and 0.1 mM-phenylmethanesulphonyl fluoride). The cells were swelled by the addition of up to 30 ml of distilled water containing 0.1 mM-phenylmethanesulphonyl fluoride and 10 μg of leupeptin/ml (4 °C) and were then homogenized in a tight-fitting glass Dounce homogenizer. Immediately after cell breakage, the ionic strength of the homogenate was restored by the addition of 2 ml of 3 M-KCl for every 40 ml of homogenate. The homogenate was then centrifuged at 7500 $g_{av.}$ for 10 s to give a supernatant (S_1) and pellet (P_1). Pellet P_1 was washed in the Tes buffer containing 10 μg of leupeptin/ml and was then treated with DNAase as described by Voorheis *et al.* (1979). The pellet (P_2), collected by centrifugation (as above), was resuspended in 40% (w/v) sucrose in Tes buffer and centrifuged at 70000 $g_{av.}$ for 3 h on a linear 40–60% sucrose gradient in the same buffer. Plasma-membrane sheets comprised the most prominent dense band; these were removed, washed three times in Tes buffer and finally resuspended in 50 mM-potassium phosphate (pH 7.0)/2 mM-EDTA. Assays of dihydrolipoamide dehydrogenase and malate dehydrogenase were carried out on the day of the preparation.

Preparation of Triton extract

Plasma membranes were incubated with 0.5% (w/v) Triton X-100 in 10 mM-Tris/HCl buffer, pH 7.4, for 20 min at 4 °C. After centrifugation at 20000 g for 60 min, the supernatant was treated with Bio-Beads SM-2 for 16 h (0.3 g wet wt. of beads/ml) at 4 °C.

Protein determination

Protein was measured by the method of Markwell *et al.* (1981). Interference by the presence of Triton X-100 was overcome by the addition of 3% (w/v) SDS (Carruthers & Melchior, 1984).

RESULTS

Presence of dihydrolipoamide dehydrogenase in *T. brucei*

Dihydrolipoamide dehydrogenase activity was detected in cell-free extracts of *T. brucei* at a specific activity of 0.03 μmol of NADH produced/min per mg of protein. The rate of production of NADH was directly proportional to the amount of enzyme extract in the assay, and all activity was destroyed on boiling the extract for 10 min. The enzyme was specific for NAD^+, no activity being detectable when $NADP^+$ was substituted for NAD^+. The stoichiometry of the reaction was measured by monitoring NADH production at 340 nm and by titrating unchanged dihydrolipoamide with 5,5′-dithiobis-(2-nitrobenzoic acid); 1.06 (±0.06) mol of NADH was produced/mol of dihydrolipoamide oxidized.

These data represent the first report of dihydrolipoamide dehydrogenase activity in a eukaryotic organism which lacks the NAD-linked 2-oxo acid dehydrogenase multienzyme complexes.

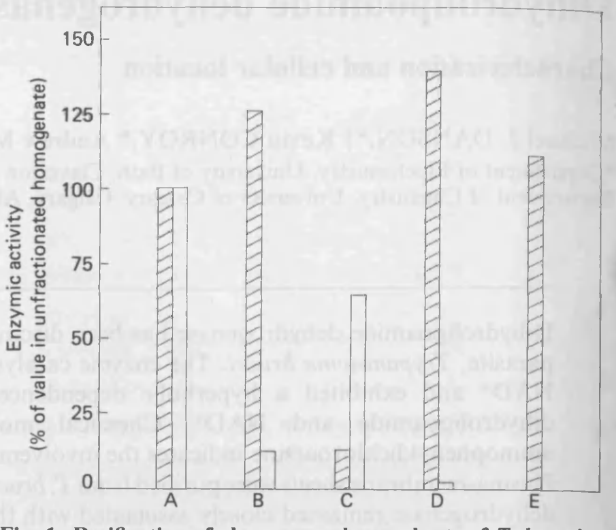

Fig. 1. Purification of plasma-membrane sheets of *T. brucei* and cellular location of dihydrolipoamide dehydrogenase and malate dehydrogenase

Plasma-membrane sheets from *T. brucei* were purified, and the enzymic activities of dihydrolipoamide dehydrogenase (▨) and malate dehydrogenase (□) were assayed, as described in the Experimental section. The enzymic activities are expressed as a percentage of their values in the unfractionated homogenate (dihydrolipoamide dehydrogenase, 0.74 μmol of NADH produced/min; malate dehydrogenase, 40.6 μmol of NADH oxidized/min). (A) Unfractionated cell homogenate; (B) pellet P_1; (C) supernatant S_1; (D) final washed pellet of plasma membranes; (E) Triton X-100 extract of purified plasma membranes, after removal of detergent with Bio-Beads.

Cellular location of dihydrolipoamide dehydrogenase

Plasma-membrane sheets were prepared from *T. brucei* by the method of Voorheis *et al.* (1979) as described in the Experimental section. The enzymic activities of dihydrolipoamide dehydrogenase and of malate dehydrogenase were measured in the homogenate, supernatant (S_1) and pellet (P_1) arising from differential centrifugation of this homogenate, and in the final washed pellet of plasma membranes. The data are given in Fig. 1. In agreement with the results reported by Voorheis *et al.* (1979), the membranes were essentially free of malate dehydrogenase, less than 2% of the original enzyme activity of the homogenate being found in the final preparation. In direct contrast (Fig. 1), the dihydro-

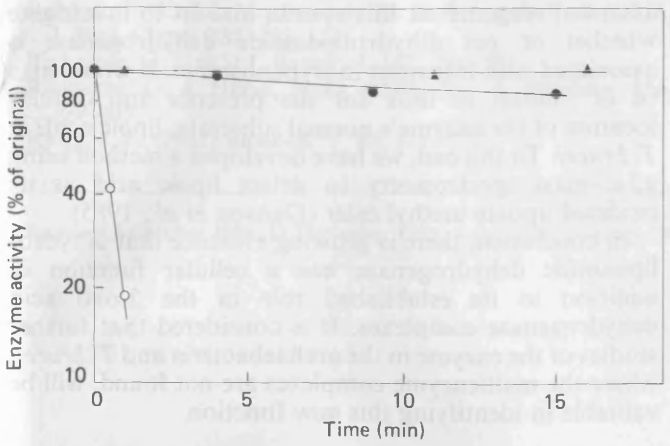

Fig. 2. Chemical modification of *T. brucei* dihydrolipoamide dehydrogenase with $H_2NPhAsCl_2$

Purified plasma membranes of *T. brucei* were incubated at 4 °C in 50 mM-potassium phosphate (pH 7.0)/2 mM-EDTA, in the presence of 0.5 mM-$H_2NPhAsCl_2$ (▲), 50 μM-NADH (●) or 0.05 mM-$H_2NPhAsCl_2$ and 50 μM-NADH (○). Dihydrolipoamide dehydrogenase was assayed spectrophotometrically at 340 nm as described in the Experimental section.

lipoamide dehydrogenase was co-purified with the plasma membranes, with only 10% of the activity being retained in the supernatant (S_1). This observation, that the enzyme is retained with the membrane sheets through a number of purification procedures and washing steps, strongly suggests a close interaction of dihydrolipoamide dehydrogenase with trypanosomal plasma membrane.

Treatment of the purified plasma membrane with 0.5% (w/v) Triton X-100 resulted in $\geqslant$ 80% of the dihydrolipoamide dehydrogenase being recovered in the supernatant after centrifugation and removal of the detergent by Bio-Beads (Fig. 1).

To provide a positive identification of the nature of the membrane in the final washed pellet, we have monitored throughout the preparation the ouabain-sensitive $Na^+ + K^+$-stimulated ATPase, which Voorheis *et al.* (1979) find to be associated with the plasma membrane. In our hands, the activity of this enzyme is both variable and very difficult to measure in the presence of comparatively large amounts of other phosphatases. Nevertheless, we find this ATPase to be retained in pellet P_1, and up to 60% of the homogenate activity to be in the final membranes. However, because of the difficulties we have encountered in assaying this enzyme, we would not wish to use these data alone as positive evidence for the presence of plasma membranes. Therefore we have subjected the final preparation to electron-microscopic examination. The purified membrane was observed to contain the pellicular microtubular array which Voorheis *et al.* (1979) state is a marker for 'the unequivocal identification of the isolated membrane material being derived exclusively from the plasma membrane'. On this basis, the plasma membrane in our preparation is confirmed.

Kinetic properties of dihydrolipoamide dehydrogenase

In both sonicated cell-free extracts and purified plasma membrane, the enzyme showed a hyperbolic dependence of rate on each substrate. The data were analysed by the direct linear plot (Eisenthal & Cornish-Bowden, 1974), and the kinetic constants determined are summarized in Table 1. There were no significant differences in K_m values between the two enzyme preparations, although the specific activity in the plasma membrane was 8-fold greater than that observed in the sonicated cell-free extracts. This increase in specific activity compares with reported values of 15-fold for ouabain-sensitive $Na^+ + K^+$-stimulated ATPase and 5-fold for adenylate cyclase (Voorheis *et al.*, 1979).

Table 1. Kinetic properties of *T. brucei* dihydrolipoamide dehydrogenase

Sonicated cell-free extracts were prepared and plasma membranes were purified as described in the Experimental section. The K_m for dihydrolipoamide was determined at a fixed NAD^+ concentration of 1 mM; the K_m for NAD^+ was determined at a fixed dihydrolipoamide concentration of Eisenthal & Cornish-Bowden (1974). Means ± S.E.M. are shown where appropriate.

	Cell-free sonicated extract	Purified plasma membranes
K_m (dihydrolipoamide)	0.18 (±0.04) mM	0.13 (±0.03) mM
K_m (NAD^+)	0.35 (±0.03) mM	0.28 (±0.05) mM
Sp. activity (μmol/min per mg of protein)	0.029	0.229

Chemical modification of dihydrolipoamide dehydrogenase with $H_2NPhAsCl_2$

The catalytic mechanism of dihydrolipoamide dehydrogenase normally involves the alternate oxidation and reduction of an intrachain disulphide bond (Williams, 1976). Thus its ligands dihydrolipoamide and NADH will render the active-site disulphide bond susceptible to dithiol-specific tervalent arsenicals (Adamson & Stevenson, 1981; Adamson *et al.*, 1984), a phenomenon which is characteristic of this enzyme and which may be used to help establish that the observed enzymic activity is the result of a true dihydrolipoamide dehydrogenase.

In accordance with the normal catalytic mechanism, the enzyme from both cell-free extracts and purified plasma membrane was rapidly inactivated by $H_2NPhAsCl_2$ in the presence of NADH (Fig. 2), whereas the reagent alone caused no significant loss of enzymic activity.

DISCUSSION

The data in the present paper constitute the first report of the presence of dihydrolipoamide dehydrogenase in *T. brucei*. The significance of this discovery stems from a number of observations. Firstly, the bloodstream form of *T. brucei* does not have the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes (Fairlamb, 1982; M. J. Danson & R. Crisp, unpublished work). In organisms which do possess these multienzyme systems, dihydrolipoamide dehydrogenase is an integral component of such complexes, and therefore its presence in

T. brucei suggests that this enzyme may have an additional metabolic role, which remains to be elucidated. This is the first time that the enzyme has been found in the absence of the 2-oxo acid dehydrogenase complexes in a eukaryotic organism, although we have previously reported a similar situation in the halophilic (Danson *et al.*, 1984, 1986), thermoacidophilic and methanogenic (Danson, 1984) archaebacteria. We have purified dihydrolipoamide dehydrogenase from *Halobacterium halobium* (Danson *et al.*, 1986) and find it to be remarkably similar to its counterpart in non-archaebacterial species in subunit arrangement and in its catalytic and kinetic properties. Similarly, in the present paper, we demonstrate that dihydrolipoamide dehydrogenase from *T. brucei* specifically catalyses the stoichiometric oxidation of dihydrolipoamide by NAD^+, and we provide evidence from chemical modification studies that a reversibly reducible disulphide bond is essential to catalysis, as has been found in other dihydrolipoamide dehydrogenases (Williams, 1976).

It should be noted that Ryley (1962) found that oxidized lipoic acid increased the rate of oxidation of NADH by homogenates of *Trypanosoma rhodesiense*, indicating the presence of dihydrolipoamide dehydrogenase in this trypanosome. However, the organism can also metabolize pyruvate to CO_2 and it may be that the enzyme is functioning in its recognized role in pyruvate oxidation in *T. rhodesiense*, a situation not occurring in *T. brucei*.

A second significant factor is that *T. brucei* dihydrolipoamide dehydrogenase appears to be located specifically in the plasma membranes of the organism. Our evidence for this comes from the co-purification of the enzyme with the plasma membrane. Moreover, in preliminary experiments, we have shown, by the method of Kasahara & Hinkle (1977), that dihydrolipoamide dehydrogenase, extracted from the purified plasma membranes with Triton X-100, can be reconstituted into soya-bean L-α-phosphatidylcholine liposomes to a specific activity the same as that in the purified membranes (K. Conroy, M. J. Danson, R. Eisenthal & G. Holman, unpublished work).

This is the first time that a eukaryotic dihydrolipoamide dehydrogenase has been reported to be membrane-associated, and such an attachment may suggest a function specific to that location. Indeed, evidence has been presented (Richarme, 1985; Richarme & Heine, 1986) that lipoic acid and dihydrolipoamide dehydrogenase may be involved in the *Escherichia coli* binding-protein-dependent transport of galactose and maltose. In this system, the enzyme is distinct from the *Lpd* gene product, which functions in the 2-oxo acid dehydrogenase complexes (Richarme & Heine, 1986). Additionally, it has been observed that the dithiol-specific tervalent arsenical phenylarsine oxide inactivates insulin-stimulated hexose transport by 3T3-L1 adipocytes (Frost & Lane, 1985). These authors noted that this inactivation of hexose transport had similar characteristics to the inactivation of 2-oxo acid dehydrogenases by the same arsenical (reacting with the lipoyl groups on the acetyltransferase component); however, the site of reaction of phenylarsine oxide with the adipocytes has not yet been defined.

An assay for the transport of glucose into *T. brucei* has now been developed (Game *et al.*, 1986). It should therefore be possible to determine the effects of tervalent arsenical reagents on this system and so to investigate whether or not dihydrolipoamide dehydrogenase is associated with transport in trypanosomes. It would also be of interest to look for the presence and cellular location of the enzyme's normal substrate, lipoic acid, in *T. brucei*. To this end, we have developed a method using g.l.c.–mass spectrometry to detect lipoic acid as its oxidized lipoate methyl ester (Danson *et al.*, 1985).

In conclusion, there is growing evidence that dihydrolipoamide dehydrogenase has a cellular function in addition to its established role in the 2-oxo acid dehydrogenase complexes. It is considered that further studies of the enzyme in the archaebacteria and *T. brucei*, where the multienzyme complexes are not found, will be valuable in identifying this new function.

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deposition. Our failure to demonstrate any reduction in the amount of mixed muscle protein does not preclude the possibility that chronic ethanol ingestion in rats may reduce the amount of specific myofibrillar proteins as suggested in alcoholic patients (Martin *et al.*, 1984).

In conclusion, the chronically treated ethanol-fed rat is a suitable model to study skeletal muscle myopathy. Anatomically distinct muscles provide a useful basis to examine types I and II fibres and demonstrate contrasting responses to treatment.

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Sugar transport in *Trypanosoma brucei*

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The bloodstream form of *Trypanosoma brucei* is dependent on glucose metabolism for energy production. Glycolysis is thought to be localized mainly if not totally in the glycosome (Opperdoes & Borst, 1977) and much has been reported on the functional features of this organelle (Opperdoes, 1985). Much less attention has been directed at the initial step of glucose metabolism, i.e. the transport of sugar across the plasma membrane. The results of Gruenberg *et al.* (1978) indicated that glucose transport is likely to be the rate-limiting step in glucose metabolism, but those studies involved the use of 2-deoxyglucose which is known to be a substrate for hexokinase. Recently Game *et al.* (1986) reported that 1-deoxy-D-glucose may be a suitable analogue for studying sugar transport in *T. brucei*. Their results on 1-deoxy-D-glucose influx demonstrated that the V for trypanosome sugar transport is slower than that for mammalian systems. However, attempts to characterize efflux and exchange kinetics of 1-deoxy-D-glucose effect were frustrated by a slow but significant phosphorylation of the analogue.

Game *et al.* (1986) also reported that 6-deoxy-D-glucose was an effective inhibitor of 1-deoxy-D-glucose uptake. As this analogue is unlikely to undergo any metabolism, we have investigated the kinetics of its uptake and efflux in intact cells and have also studied D-glucose transport in vesicles prepared from trypanosome plasma membranes.

6-Deoxy-D-glucose was synthesized from 6-chloro-α-methyl-D-glucoside (Evans & Parrish, 1972) which was converted to the 6-iodo compound and then catalytically reduced with tritium gas (Amersham). This tritiated product was hydrolysed to remove the glycosidic methyl group and the resultant [6-^{3}H]deoxy-D-glucose was then purified by paper chromatography.

Kinetic parameters for 6-deoxy-D-glucose were determined at 20°C. Net influx and efflux showed similar K_m and V_{max} values (Table 1) indicating the transport system is kinetically symmetric. An equilibrium exchange experiment in which isotope exchange flux was studied in cells pre-equilibrated with non-labelled 6-deoxy-D-glucose also showed comparable kinetic parameters (Table 1). Thus the system does not show accelerated exchange. An integrated rate equation was used to determine the infinite-*cis* K_m. This experiment confirmed kinetic symmetry for the transport of 6-deoxy-D-glucose.

We have also studied hexose transport in purified plasma membranes. Vesicles were prepared by a freeze-thaw procedure. The freeze-thaw protocol was found to be essential to remove cytoskeletal components associated with the plasma membrane sheets. We have found that an infinite-*trans* protocol is most useful for studying the kinetics of transport in this isolated membrane system. In this procedure vesicles are loaded with 100 mM-D-glucose and then diluted into a buffer containing radiolabelled D-glucose at a range of concentrations. The high concentration of D-glucose inside traps (by counterflow) the radiolabelled D-glucose as it enters the vesicles. The estimated K_m is shown in Table 1. The K_m of 14 mM is considerably higher than the affinity constant estimated by using intact cells and further experiments will investigate the reasons for the difference.

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Table 1. *Kinetic constants for sugar transport in T. brucei*

Substrate	Procedure	K_m (mM)	V_{max} (mM s^{-1})
6-Deoxy-D-glucose	Zero-*trans* entry	2.17 ±0.068	0.279 ±0.006
6-Deoxy-D-glucose	Zero-*trans* exit	6.063 ± 1.227	0.863 ± 0.109
6-Deoxy-D-glucose	Infinite-*cis* entry	6.212 ± 1.197	0.601 ± 0.052
6-Deoxy-D-glucose	Equilibrium exchange	3.0 ± 0.332	0.965 ± 0.067
D-Glucose	Infinite-*trans* entry in vesicles	14.33 ± 2.55	–

Proteolytic degradation of mucus in the colon

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A continuous adherent layer of insoluble mucus gel is found on the colonic mucosal surface, mean thickness 150 ± 110 μm in the rat (Sakata & Engelhardt, 1981). Such a mucuos layer will provide a protective barrier against harmful agents in the lumen and mechanical damage from motile forces of digestion. The protective gel layer is formed by polymeric glycoproteins which are fragmented by proteases to dissolve the mucous layer (mucolysis) (Allen *et al.*, 1984; Bell *et al.*, 1985). Recent studies of proteolytic activity in stools (using non-mucous protein substrates) showed that inflammatory bowel disease patients had significantly higher levels of activity compared to non-symptomatic controls (Corfield *et al.*, 1986). If such proteolytic activity reflects excessive mucolytic activity (mucous degrading) then this may be an important factor in disruption of the colonic mucosal barrier in inflammatory bowel disease. Here we demonstrate mucolytic activity in human faecal extracts from non-inflammatory bowel disease patients.

Glycoproteins from pig and human colonic mucous and pig gastric mucuous were extracted in proteinase inhibitors [1.0 mM-phenylmethylsulphonyl fluoride (PMSF), 50 mM-iodoacetamide, 100 mM-α-aminohexanoic acid, 5 mM-benzamidine HCl, 1.0 mg l^{-1} soybean trypsin inhibitor and 10 mM-Na_2 EDTA in 0.5 M-Tris/HCl pH 8.0] and purified by two successive equilibrium centrifugation steps in 3.5 M-$CaCl_2$. Purified glycoproteins were largely excluded (> 60%) from Sepharose CL-2B and were fragmented into smaller species, included on Sepharose CL-2B, by reduction (0.2 M-mercaptoethanol 48 h, 4°C) and exhaustive proteolysis [1 : 100 (w/w) papain : glycoprotein, 48 h, 60°C]. This fragmentation was reflected by a substantial drop in solution viscosity (approximately 10-fold on papain digestion of pig colonic mucous glycoprotein at 4 mg ml^{-1}).

Human faecal extracts (from non-inflammatory bowel disease patients) were obtained by suspending samples of stool in 4 vol. of M/15 M phosphate buffer pH 7.5, containing 50 mM-NaCl and centrifuging at 10 000 ***g*** for 15 min at 4°C. Protease activity was measured by a sensitive trinitrobenzene sulphonic acid assay for formation of new peptide *N*-terminals at pH 7.5 and 37°C using succinyl albumin or purified mucous glycoprotein as substrate (Hutton *et al.*, 1986). Protolytic action on succinyl albumin by the extracts was inhibited by soybean trypsin inhibitor and PMSF (1 mM) [but not by iodoacetamide (50 mM) or Na_2 EDTA (10 mM)] suggesting that the activity was due to a trypsin-like serine-dependent protease. Subsequently, levels of faecal protease activity (against succinyl albumin) were quantified as equivalents of porcine pancreatic trypsin (Sigma) activity by weight. On this basis, faecal protease activity was completely inhibited by soybean trypsin inhibitor [1 : 1 (w/w) inhibitor : faecal protease activity] and the synthetic polyacrylate Carbomer 934P [1000 : 1 (w/w) polyacrylate : faecal protease activity]. PMSF [100 : 1 (w/w)] inhibited activity by ~60%. Mucolytic activity of the faecal extracts was assayed by monitoring the fall in specific viscosity of solutions of purified mucous glycoproteins, as a function of time, on incubation with faecal extract at pH 7.5 and 37°C. Mucolysis was also followed by measuring the increase in free *N*-terminals and the amount of glycoprotein included in Sepharose CL-2B. In this manner, extracts were shown to proteolytically digest purified pig gastric and colonic mucous glycoproteins. For example, incubation of purified pig colonic muous glycoprotein with extract [0.1% enzyme : glycoprotein (w/w)] induced a rapid fall in the specific viscosity of the glycoprotein in the first 15 min of digestion (74.5% of the fall in specific viscosity after complete digestion) followed by a slower fall over the subsequent 48 h. This fall in viscosity was accompanied by an increase in the proportion of glycoprotein included on Sepharose CL-2B this being 35% at time 0, and 77% and 89% after 15 min and 24 h, respectively. Decrease in viscosity was also mirrored by an increase in the number of free *N*-terminals and a 50% drop in specific viscosity corresponded to 3.1×10^{-5} mol of peptide bonds cleaved. The synthetic polyacrylate Carbomer 934P [1200 : 1 (w/w) polyacrylate : faecal protease activity] inhibited mucolysis by 45%.

The polyacrylate Carbomer 934P, in addition to inhibiting degradation of mucus, was shown to have a potential role in strengthening the protective mucous barrier. Carbomer 934P synergistically increased the viscosity of purified undegraded colonic mucous glycoproteins from both man and pig. Thus, viscosity of glycoprotein/carbomer mixtures (GC) was substantially greater than the sum of the individual viscosities (G+C). This synergistic increase in viscosity was approximately 460% at concentrations of glycoprotein and carbomer of 4 mg ml^{-1} and 2 mg ml^{-1}, respectively.

These studies demonstrate endogenous mucolytic activity, by proteases, exists in the lumen of the colon *in vivo*. Such mucolysis may be an important factor in disruption of the colonic mucous barrier in disease.

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Abbreviation used: PMSF, phenylmethyl sulphonyl fluoride.

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